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14. ABSTRACT

Calreticulin (CALR) is known to be a major player in the ER quality control of glycosylated proteins. Remarkably, this ubiquitously expressed housekeeping gene is mutated in ~30% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF), 2 of the 8 major myeloproliferative neoplasms (MPNs) as classified by the world health organization. One of the biggest challenges is to understand how Jak2, MPL and CALR mutant proteins can lead to very similar signaling events and phenotypes in these MPNs.

Objective/Hypothesis. We hypothesize that ET and PMF phenotypes result from abnormal signaling and aberrant intracellular trafficking events in cells bearing mutations in JAK2, MPL or CALR. In this project, we will acquire a deeper understanding of the interplay between these three gene products. We will compare and contrast intracellular routing for wildtype and mutated proteins, identify their potential intersections, and determine the cellular locations from which signals are propagated. Our work seeks a broader understanding of the pathogenesis of the MPNs and set the stage for development of targeted therapeutic solutions.

Our Specific Aims are:

Aim 1. To determine the sub-cellular localization of mutant calreticulin, as well as the impact on MPL trafficking and megakaryocyte (MK) ultrastructure.

Aim 2. To evaluate mechanisms underlying aberrant signaling in MPNs bearing mutant CALR or MPL.

At first, we focused on building a MPN sample bank in order to gain access to primary patient's samples carrying *JAK2*, *MPL* or *CALR* mutations. These primary samples were then used to study in depth intra-cellular trafficking and signaling patterns of mutants *MPL* and *CALR*.

We then focused our efforts on mutations in *MPL* that can drastically impair its function and be a contributing factor in multiple hematologic malignancies, including congenital amegakaryocytic thrombocytopenia (CAMT), and we reported a unique familial cases of CAMT presenting with a previously unreported *MPL* mutation: T814C (W272R) in the background of the activating *MPL* G117T (K39N or Baltimore) mutation. Confocal microscopy, proliferation and surface biotinylation assays, as well as co-immunoprecipitation and western blotting analysis were used to elucidate the function and trafficking of Mpl mutants. Results showed that Mpl protein bearing the W272R mutation, alone or together with the K39N mutation, lack detectable surface expression while being strongly co-localized with endoplasmic reticulum (ER) marker, calreticulin. Both WT and K39N-mutated Mpl were found signaling competent, while single or double mutants bearing W272R were unresponsive to Tpo. Function of the deficient Mpl receptor could be rescued using two separate approaches: 1) GRASP55 over-expression, which partially restored Tpo-induced signaling of mutant Mpl by activating an autophagy-dependent secretory pathway and, thus, forcing ER-trapped, immature, receptors to traffic to the cell surface; and 2) CRISPR-Cas9 gene editing used to repair *MPL* T814C mutation in transfected cell lines and primary umbilical cord blood-derived $CD34^+$ cells. We demonstrated proof-of-principle for rescue of mutant Mpl function using gene editing of primary hematopoietic stem cells, indicating direct therapeutic applications for CAMT patients.

Finally, we dissected a unique case of post-ET MF with AML transformation, presenting with previously unreported double mutations: *MPL*W515R and a *CALR* 52-base pair deletion (Type I). Our study revealed that subsequent acquisition of *MPL*W515R by the MPN clone occurred temporally with AML transformation. Mechanistic studies using gene-edited UT-7 cell models are still ongoing. This study will provide important insight in the understanding of MPNs progression and the relative contribution of *CALR* and *MPL* mutations to neoplastic transformation.

15. SUBJECT TERMS

None provided

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1. Introduction

Calreticulin (CALR) is known to be a major player in the ER quality control of glycosylated proteins. Remarkably, this ubiquitously expressed housekeeping gene is mutated in ~30% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF), two of the 8 major myeloproliferative neoplasms (MPNs) as classified by the WHO. One of the biggest challenges is to understand how Jak2, MPL and CALR mutant proteins can lead to very similar signaling events and phenotypes in these MPNs. We hypothesize that ET and PMF phenotypes result from abnormal signaling and aberrant intracellular trafficking events in cells bearing mutations in *JAK2, MPL* or *CALR*. In this project, we will acquire a deeper understanding of the interplay between these three gene products. We will compare and contrast intracellular routing for wildtype and mutated proteins, identify their potential intersections, and determine the cellular locations from which signals are propagated. Our work seeks a broader understanding of the pathogenesis of the MPNs and set the stage for development of targeted therapeutic solutions.

2. Keywords

- Thrombopoietin Receptor (Mpl)
- Janus kinase 2 (Jak2)
- Calreticulin (CALR)
- Myeloproliferative Neoplasms (MPN)
- Essential Thrombocythemia (ET)
- Congenital Amegakaryocytic Thrombocytopenia (CAMT)
- Genome editing

3. Accomplishments

a. What were the major goals of the project?

- To determine the sub-cellular localization of mutant calreticulin, as well as the impact on Mpl trafficking and megakaryocyte (MK) ultrastructure.
- To evaluate mechanisms underlying aberrant signaling in MPNs bearing mutant CALR or Mpl.

b. What was accomplished under these goals?

Establishing the UT-7/Tpo sub-line. In order to establish the UT-7/Tpo sub-line, the UT-7 cells have been grown at sub-optimal concentrations of thrombopoietin (Tpo) and in the absence of other cytokines. Expression of Mpl in the Tpo-selected cells was assessed by western blotting (WB). This new cell line has then been sub-cloned and individual clones were screened for robust Mpl expression using WB before being further modified (**Fig. 1**).

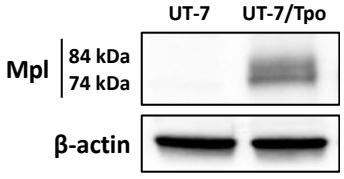


Figure 1. Mpl expression level in UT-7 and Tpo-selected UT-7 cells, UT-7/Tpo.

CRISPR-engineering of UT-7/Tpo cells. Our first goal has been to introduce the most common calreticulin (CRT) mutation (Type 1 or T1) found in MPNs into UT-7/Tpo cells using genetic engineering. The left panel in **Fig.2** show the presence of a sub-population of UT-7/Tpo cells that express the T1 mutant form of calreticulin after the genome editing experiment. Sub-cloning of these cells allowed us to identify edited cells that are homozygous for this mutation, as shown by PCR screening shown in the right panel of **Fig. 2**. Further editing of these cells in order to establish a unique model system for MPN studies to introduce fluorescent tags for calreticulin and/or Mpl are ongoing.

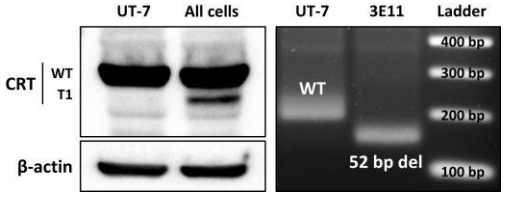


Figure 2. Introduction of calreticulin T1 mutation in UT-7/Tpo cells using genome engineering.

Proximity Ligation Assay on Mpl and wildtype, or mutant, calreticulin. In order to determine the differential interaction between the TPO receptor, Mpl, and its chaperone, calreticulin, in its wildtype or mutant form, we decided to implement an experimental procedure called DuoLink. This assay was performed on primary hematopoietic CD34+ cells that were expressing Mpl WT and both T1 mutant and WT calreticulin. Antibodies specific for either WT or mutant calreticulin allowed us to discriminate interactions of either protein with Mpl WT in these cells. Results presented in **Fig. 3** (top panel) show an ER-like distribution of interactions between Mpl WT and calreticulin WT. Striking differences were observed when analyzing interactions between Mpl WT and mutant T1 calreticulin. These interactions were less abundant and differentially localized near the plasma membrane. We also noticed that the shape of the fluorescent complex formed during the assay was more elongated when Mpl WT interacted with mutant T1 calreticulin than calreticulin WT, indicating a possible change in protein distribution in this sub-cellular compartment, which has not yet been identified.

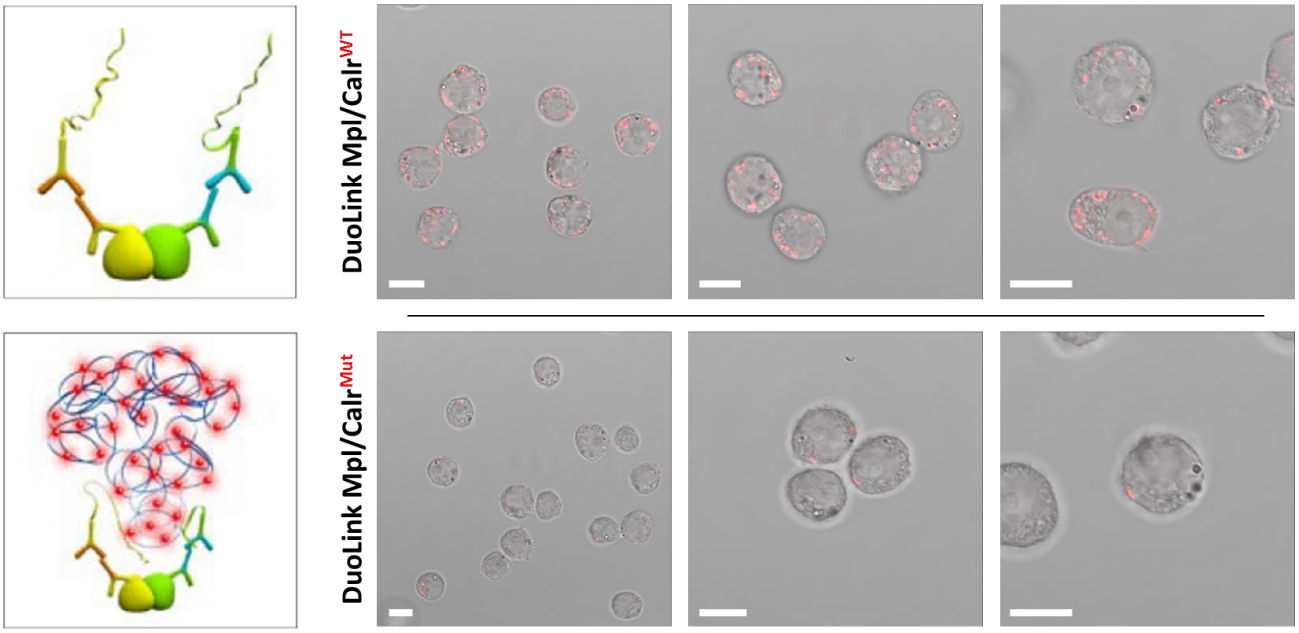


Figure 3. Proximity ligation/microscopy assay performed on CD34+ cells showed that Mpl interacts with mutant calreticulin in a different cellular compartment, outside the ER and near the plasma membrane, than with wildtype calreticulin (Scale bars = 5 µm).

Studies of a novel Double Mpl Mutation. It is important to note that, while activating mutations in these genes result in thrombocytosis (too *many* platelets); there are also distinct mutations that result in thrombocytopenia (too *few* platelets). During this initial reporting period, we were able to study a new double Mpl mutation that we discovered (in collaboration with our colleagues in Nimes, France). In this case report of 3 siblings with congenital amegakaryocytic thrombocytopenia (CAMT), we show that the Mpl gene had two mutations in tandem – both an activating mutation (Mpl K39N) and an inactivating mutation (W272R). Because of three children in this family had severely low platelet function (including one fatality), this was an amazing opportunity to study how the inactivating mutation was able to override the gain-of-function mutation. We used our powerful tools for imaging and genome editing to study the intracellular trafficking of both singly- and doubly-mutated Mpl, fitting well with Aim 2 of this project.

Highlights from the new manuscript showed this novel mutation results in entrapment of the mutant Mpl in the ER, where it is co-localized with wildtype CALR. Thus, aberrant trafficking of Mpl is responsible for the absence of proper signaling and thus, the phenotype of severe thrombocytopenia in affected patients (**Fig. 4**). Preliminary data on this CAMT family was provided in the grant application and we are very pleased that this study could be initiated and completed during the first reporting period.

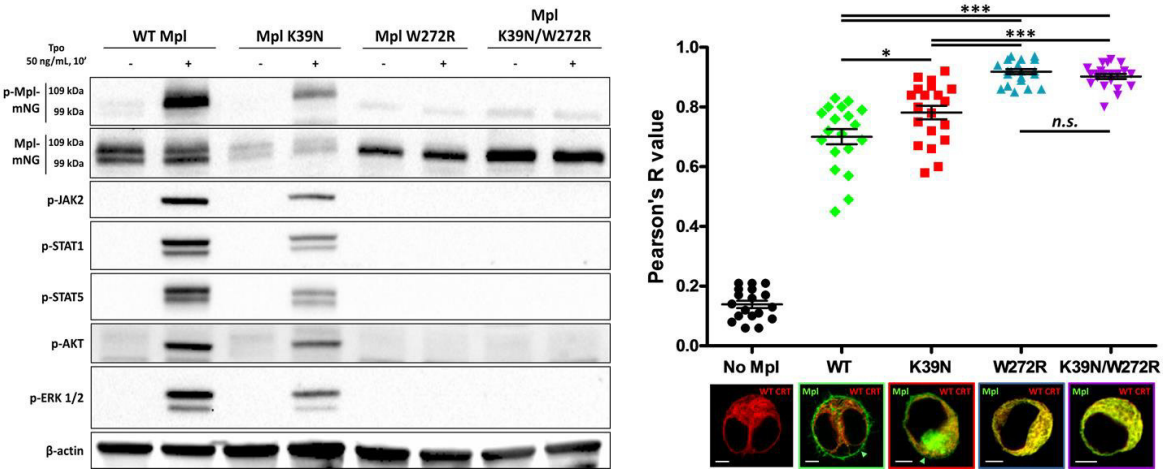


Figure 4. Aberrant intracellular trafficking of Mpl is responsible for absence of response to its ligand, Tpo, in UT-7 cell lines. At left, we evaluated Tpo signaling through the JAK/STAT, MAPK and PI3K pathways. Both WT and K39N-mutated Mpl were competent for signaling, while single or double mutants bearing W272R were unresponsive to Tpo. At right, Co-expression of WT CALR fused to RFP (ER marker) showed significantly higher co-localization (R value) with mutant Mpl than with WT Mpl, evidence that most receptors were retained within the ER.

Additional data from this new manuscript are shown in **Figures 5-7,** addressing our stated goal to develop new targeted therapeutic solutions. Our goal was to establish "proof-of-principle" for approaches that might functionally rescue the mutated thrombopoietin receptor. We previously showed that an autophagy-dependent, "alternative" secretory pathway can allow the ER form of Mpl to reach the plasma membrane. By overexpressing GRASP55, we showed that activation of this secondary trafficking pathway was successful in rescuing part of Mpl function and response to Tpo (**Fig. 5**).

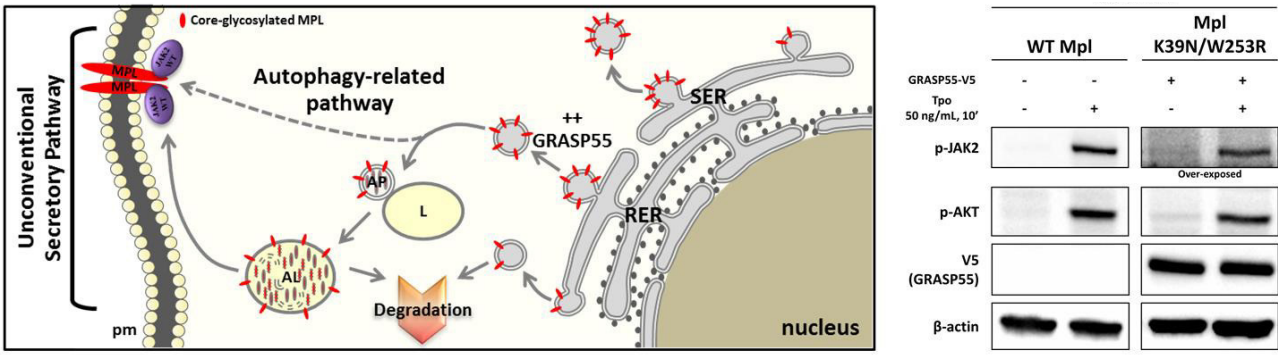


Figure 5. Tpo-induced signaling was partially restored via GRASP55 over-expression, forcing ER-trapped Mpl to traffic to the cell surface using unconventional secretion. Left panel adapted from Cleyrat *et al.* Traffic 2014.

Results were even more striking when using genome editing to restore the wildtype sequence of the gene coding for Mpl in order to fully rescue its function and response to its ligand (**Fig. 6**). Genetic editing (using CRISPR/Cas9) performed on cells carrying the W272R mutation restored the WT sequence and the response to Tpo ([Tpo] = 3 ng/mL), with similar cell proliferation as WT Mpl cells.

Thus far, our study of a newly discovered mutation of Mpl (W272R) that effects the trafficking and signaling of Mpl has led to important findings on Mpl sub-cellular trafficking and its impact on basic cell signaling. Function of the deficient Mpl receptor could be rescued using two separate approaches: CRISPR/Cas9 genetic engineering and GRASP55 over-expression. These results add to our understanding of Mpl multiple roles in the pathogenic mechanisms of several myeloid malignancies and significantly increase the relevance of our upcoming experiments using the CRISPR-edited UT-7/Tpo cell lines.

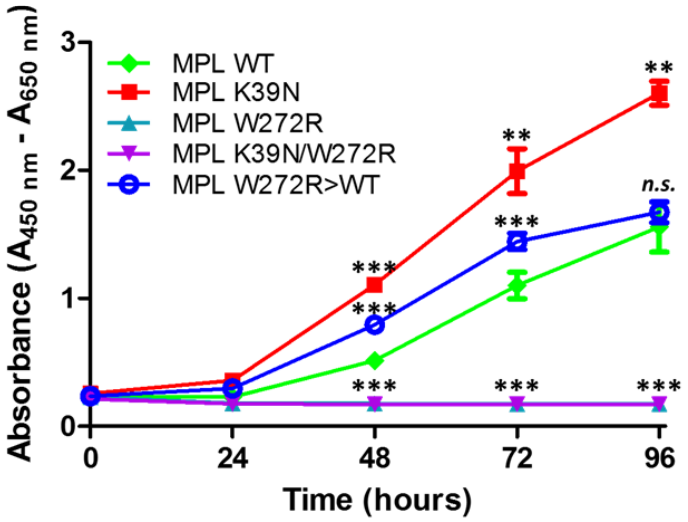


Figure 6. Functional rescue of mutant Mpl (Mpl W272R>WT) using genome editing.

To test our gene editing tools we next applied our gRNA/WT Cas9 editing approach to human K562 cells and primary hematopoietic stem and progenitor cells (HSPCs). Our goals were to confirm targeting of the *MPL* locus and to demonstrate the potential to restore megakaryocytic progenitor capabilities in CAMT patient-derived CD34$^+$ cells. To test the specificity of our gRNAs, we conducted an in vitro cutting assay using WT or WR templates. Results presented in **Fig. 7B** show that both gRNAs in the presence of their respective templates exert a very good, and similar, cutting efficiency nearing 80% (quantified in **Fig. 7C**, left panel). We then compared two different CRISPR-Cas9 delivery methods, plasmid DNA and ribonucleoproteins (RNPs), to edit K562 cells and HD cord blood (CB) CD34$^+$ cells. **Fig. 7C**, right panel, shows that gRNA #1WT performed equally as well when delivered either as plasmid DNA or RNP in K562 cells. Both delivery systems were also found comparable in inducing indels formation when editing CB CD34$^+$, with a 4 to 6-fold decrease in efficiency compared to editing in K562 cells (~50% and ~10% of indels, respectively).

Our gene editing approach was next applied to primary HSPCs isolated from the umbilical cord blood of patient II.4. **Fig. 7D** shows the sequencing results of un-edited (top panels) and edited (lower panels) cells at day 5 post-editing. The homozygous K39N mutation could be found in both populations, as expected. However, in the edited cell population, the W272R mutation appeared to be mostly heterozygous. We conclude that most of the edited cells contained only one modified copy of *MPL*, reverting the sequences from R272 to W272.

We used two different assays to show that only one functional allele was required for edited cells to deliver functional Mpl to the cell surface and proliferate in the presence of Tpo. Receptors on the surface of edited cells were detected using a fluorescently labeled anti-Mpl antibody and quantified by flow cytometry. As shown in **Fig. 7E**, control CD34$^+$ and II.4 edited CD34$^+$ displayed similar amount of surface Mpl proteins, while non-edited II.4 CD34$^+$ did not bind Mpl antibodies. We specifically selected for cells with surface expression of Mpl, by maintaining cultures before and after editing in high Tpo conditions (100 ng/mL) as the sole growth factor.

Finally, single colony assay results in **Fig. 7F** demonstrated that edited CD34$^+$ cells from patient II.4 were able to generate a significantly higher number of megakaryocytic colonies in the presence of Tpo than non-edited cells from this patient and compared to control CB CD34$^+$. Results observed in this experiment reflect both the presence of efficiently edited cells, as well as cells bearing alternate *MPL* exon 5 sequences that restored functionality.

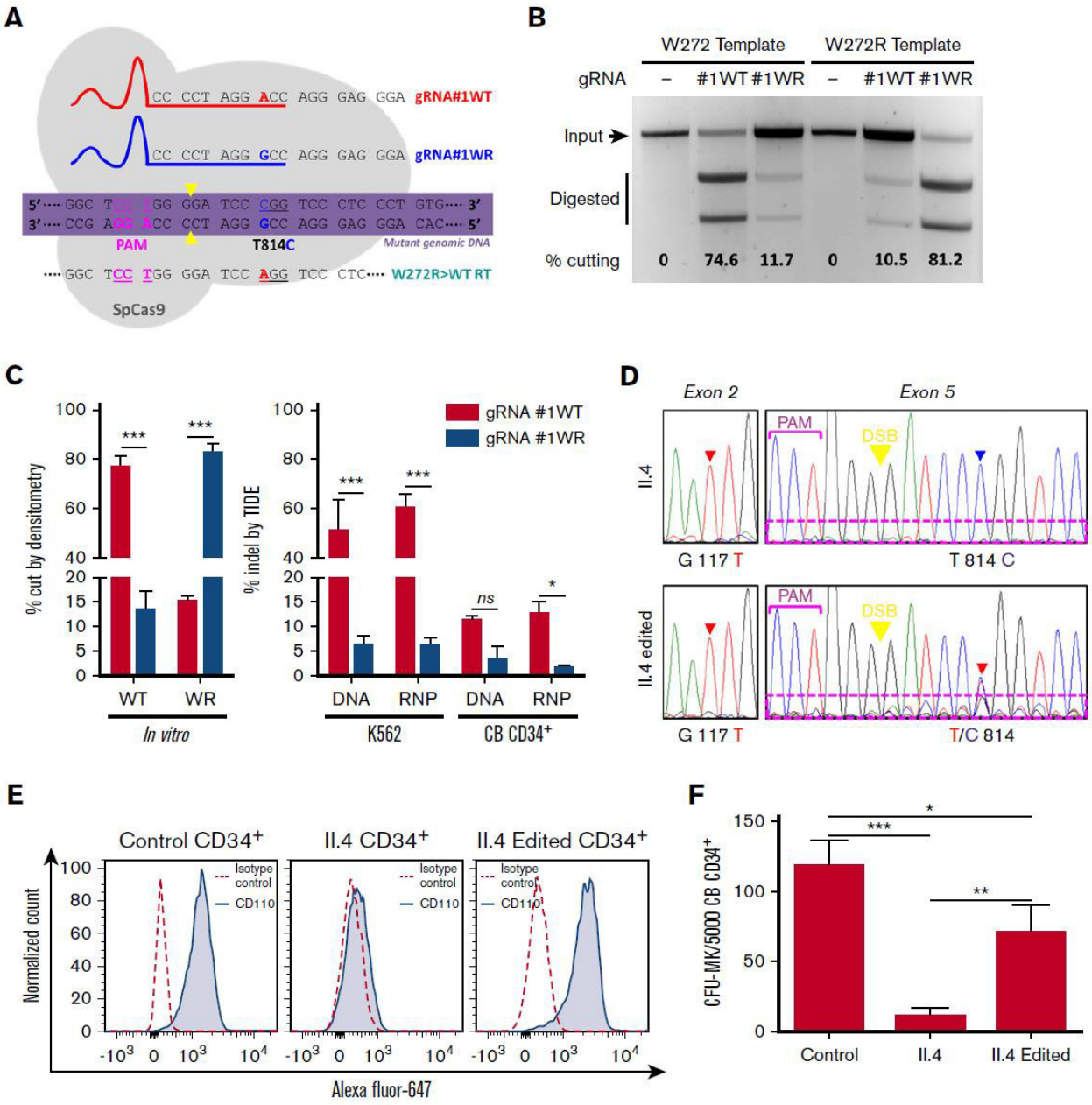
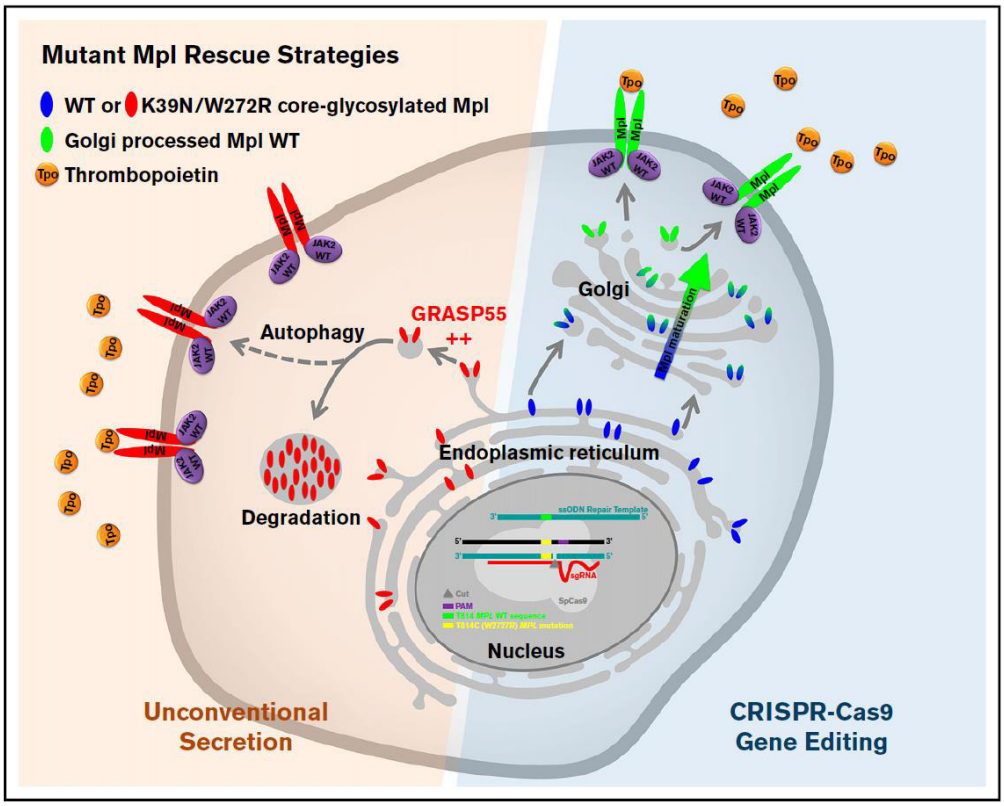


Figure 7. Gene editing in K562 cells and primary CD34+ cells. (A) Schematic of sequence-specific gRNA#1WT and gRNA#1WR and their target genomic MPL sequence representing the protospacer-adjacent motif (PAM), double-strand break site (DSB) (yellow arrowheads), and the W272R single point mutation site (T814C). DNA codons are underlined, and the repair template (RT) used to convert the W272R mutation to the WT sequence (W272R.WT) is also represented. (B) Example of in vitro digestion assay with gRNA#1WT or gRNA#1WR in the presence of their match or mismatch target sequences. Quantification of cutting efficiency was performed by using densitometry analysis. (C) Left panel shows quantification of in vitro cutting capabilities of gRNA#1WT and gRNA#1WR. Right panel shows quantification of the percentage of indel formation obtained with gRNA#1WT and gRNA#1WR when delivered as plasmid DNA or RNP complexes in K562 or CB CD341 cells. (D) Control, unedited, and edited CD341 cells isolated from patient II.4 were sequenced at day 5 after editing. G117T represents the K39N mutation and T814C represents the W272R mutation. Dotted magenta rectangles highlight the presence of additional overlapping sequences in edited cells for the T814C locus, indicating an off-target effect. (E) Flow cytometry analysis of anti-Mpl (CD110)-AF-647 binding on control CD341 cells, unedited patient II.4 CD341 cells, or edited II.4 CD341 cells at day 5 after editing. (F) In vitro megakaryocytic colony formation assay conducted in the presence of Tpo with the same cell samples used in panel E. *P , .05; **P , .005; ***P , .0001. CFU.

Altogether, we can summarize the results of this study with the diagram presented in **Fig. 8** (below). It shows the two rescue approaches used to restore Mpl function: 1) over-expression of GRASP55 to force immature Mpl receptor expression at the cell surface using unconventional, autophagy-dependent, secretion; 2) CRISPR-Cas9 gene editing to convert mutated Mpl DNA sequence to WT sequence.



Establishing a pipeline of MPN patient samples. Our IRB-approved protocol (HRRC#05-435) to consent and collect blood and bone marrow from MPN patients opened in January 2016. Enrollment has been excellent, with 85 samples collected and processed. The most common MPN mutations are well represented in this cohort of patients thus far. An important first step has been to optimize and streamline our sample processing protocols in order to standardize sample collection and subsequent banking. **Fig. 9** represents the final optimized process.

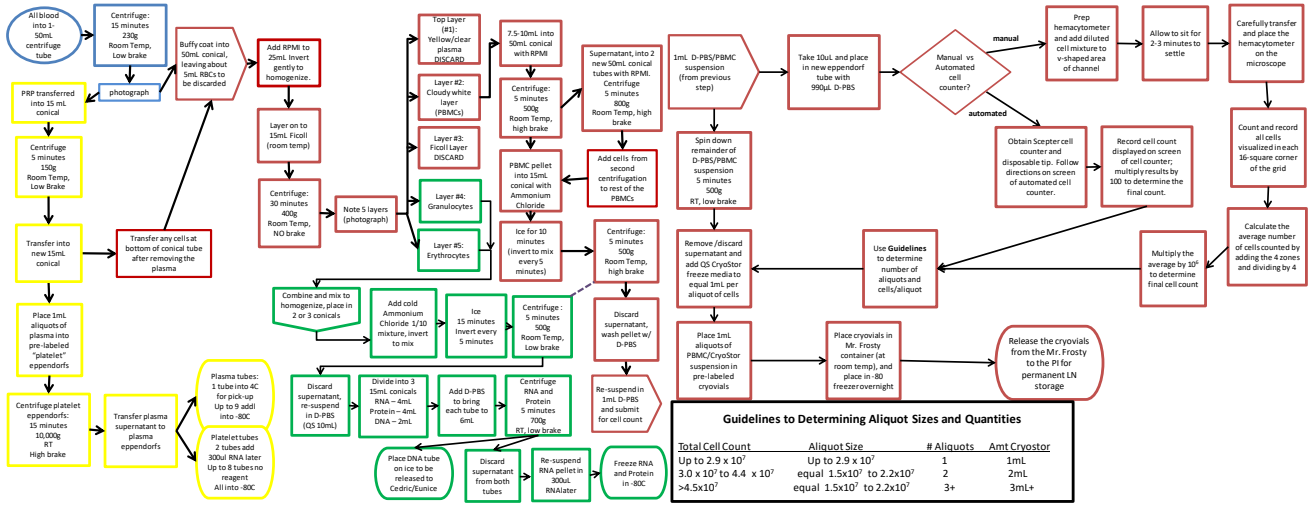


Figure 9. Optimized blood and bone marrow processing and banking chart.

We also took this opportunity to refine our methods for generating megakaryocytic colonies from progenitor cells in these samples. An example of *in vitro* CD34^{+}-derived and pro-platelet-forming megakaryocytes grown from patient's samples are shown in **Fig. 10**.

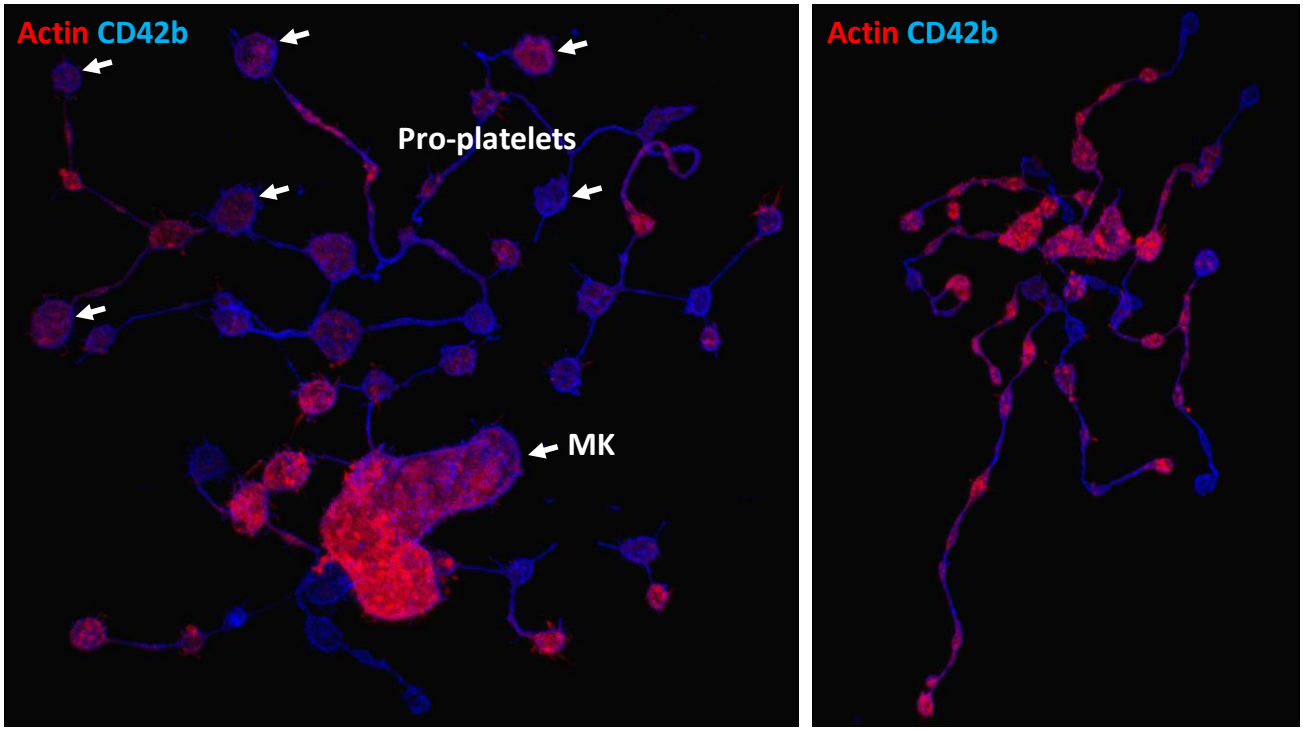


Figure 10. *In vitro* differentiated megakaryocytes from CD34^{+} primary cells after 19 days in culture without cytokines. Patient was *JAK2*V617F positive.

Leukemic Transformation of Post-Essential Thrombocythemia Myelofibrosis: A Unique Case Presenting With Double MPL and CALR Mutations. The analysis of one of our study patient revealed an intriguing and highly unusual case of case of post-ET MF with AML transformation, presenting with previously unreported double mutations: *MPL*W515R and a *CALR* 52-base pair deletion (Type I).

The case report is as follow. A 69 year-old female was diagnosed with ET in 1996, and subsequently progressed to post-ET MF in 2008. Molecular studies in 2008 were negative for *JAK2*V617F and the *BCR*/*ABL1* fusion. At this time, the patient was treated with hydroxyurea and erythropoietin. In 2010, the patient participated in the phase III COMFORT-I trial of ruxolitinib versus placebo and was randomized to ruxolitinib. Ruxolitinib was discontinued in 2014 due to severe anemia and transfusion dependence. The patient also showed no response after 3 cycles of the phase I/II trial of PRI-724, a β-catenin/wnt pathway inhibitor. She resumed ruxolitinib in 2015, but developed worsening leukocytosis and myeloid immaturity, progressing to AML in January 2016. She stopped ruxolitinib and received azacytidine for one cycle. However, the patient developed tumor lysis syndrome and acute on chronic kidney injury and expired. Molecular studies at the time of leukemic transformation revealed *CALR* type I and *MPL*W515R mutations. A repeat test for *JAK2*V617F was negative.

We decided to further investigate this highly relevant clinical case and focus on the functional characterization of the *MPL*/*CALR* double mutations, their impact on Mpl intracellular trafficking and signaling, and their correlation with disease progression.

Results presented in the following Fig. 11-13 recapitulate the major findings thus far. This work was also presented at the 2017 ASH meeting where it was very well received. A manuscript in under preparation.

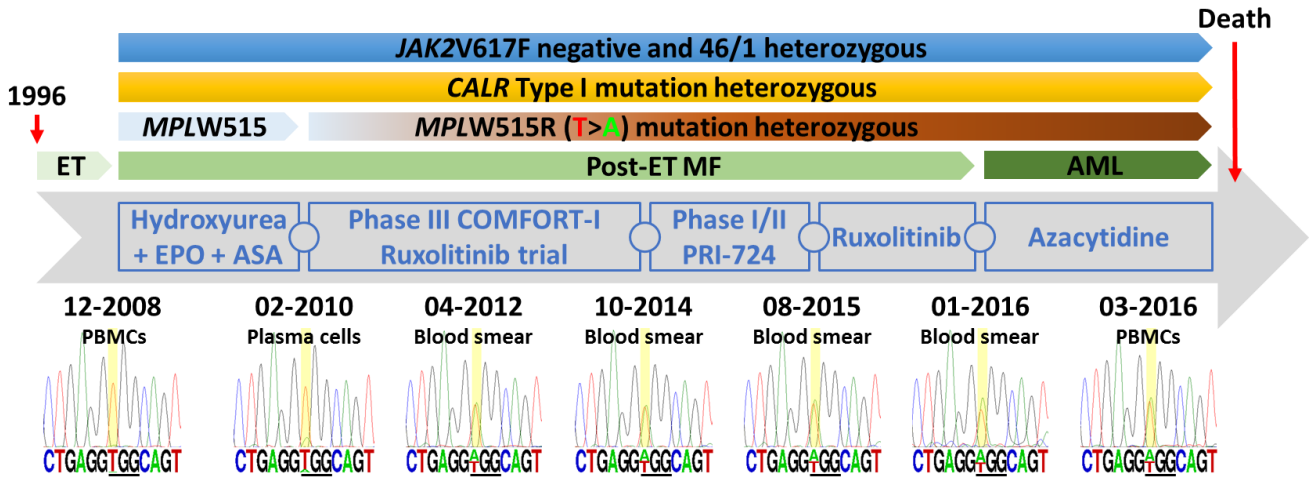


Figure 11. Timeline. Presence of heterozygous *CALR* Type I mutation at diagnosis and expanding *MPL*W515R mutation throughout progression to AML (sequencing trace profiles). $CD34^+$ blasts tested positive for *MPL*W515R while $CD3^+$ lymphocytes tested negative.

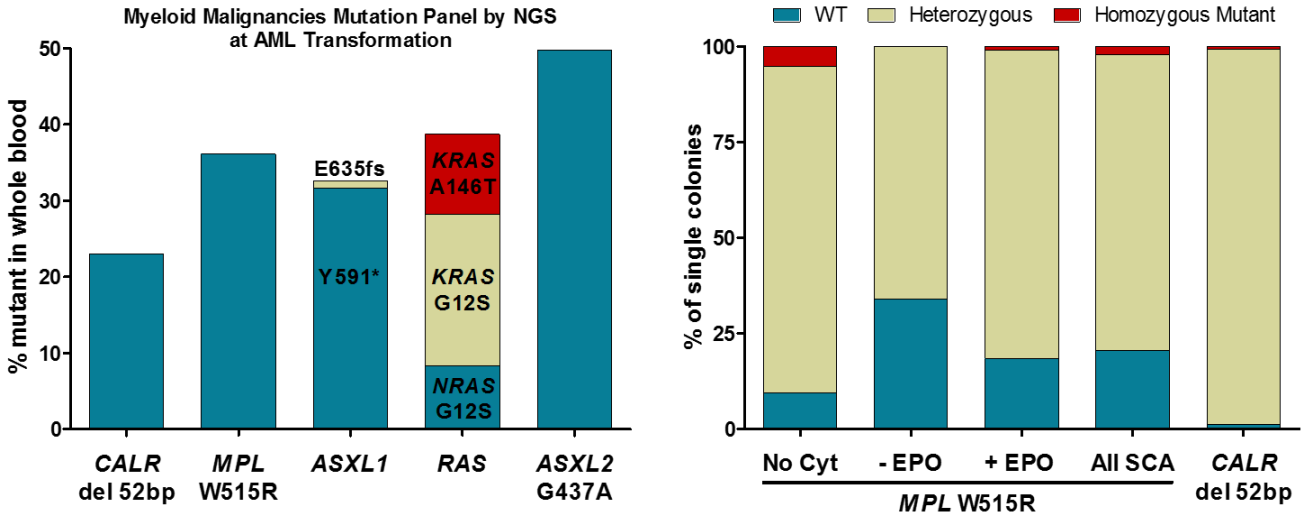


Figure 12. Myeloid Panel at left. Next-Gen sequencing revealed the presence of additional mutations of *ASXL1*, *RAS* and *ASXL2* (this is the first report of *ASXL2* G437A). Single Colony Assay at right. ~300 single colonies tested. 98.2% were $CALR^{Het}$, 1.1% $CALR^{WT}$ and 0.7% $CALR^{Mut}$. 75.3% were MPL^{Het}, 22.4% MPL^{WT} and 2.3% MPL^{Mut}.

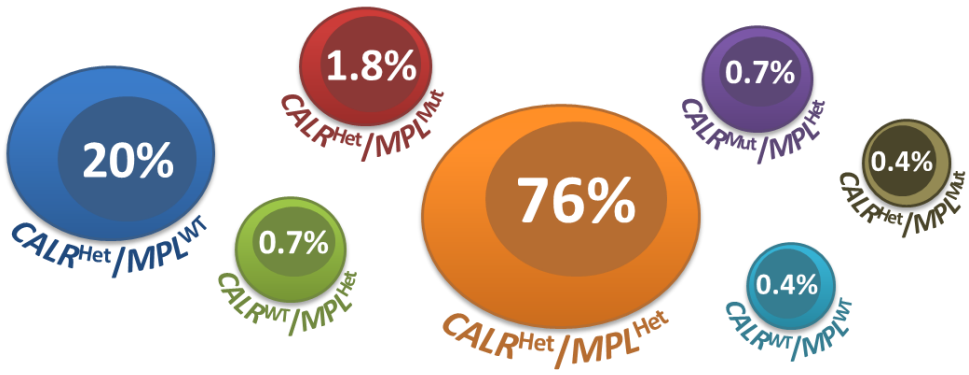


Figure 13. Representation of all malignant MPN/AML clones found in the single colony assay with *CALR* and/or *MPL* mutations.

c. **What opportunities for training and professional development has the project provided?**
Nothing to report

d. **How were the results disseminated to communities of interest?**
- Press release issued by the UNM Cancer Center generated an article in the Albuquerque Journal, as well as a local TV interview at http://cancer.unm.edu/newsroom/new-grant-helps-new-mexicans-with-blood-disorders/

e. **What do you plan to do during the next reporting period to accomplish the goals?**
Nothing to report

4. Impact

a. **What was the impact on the development of the principal discipline(s) of the project?**
Nothing to report

b. **What was the impact on other disciplines?**
Nothing to report

c. **What was the impact on technology transfer?**
Nothing to report

d. **What was the impact on society beyond science and technology?**
Nothing to report

5. Changes/Problems

a. **Changes in approach and reasons for change**

- The MPN field is moving forward rapidly, particularly in the context of CALR mutations. For example, new papers by Araki et al and Marty et al (both in the March 10, 2016 issue of *BLOOD*) demonstrated that the mutant CALR protein specifically activates Mpl. This leads to constitutive activation of the Jak2 pathway. These data from the literature strongly supports our original hypothesis for functional linkage between these three proteins. However, thus far, the location where CALR-Mpl-Jak2 signaling takes place has not been resolved. We will bring all of our tools to bear on this problem. We will also produce recombinant, mutant CALR in order to perform biochemical experiments testing the hypothesis that it acts like a "ligand". We will use our new BLITZ apparatus to evaluate binding to purified Mpl and determine the affinity of this interaction.

  These new reports motivate us to emphasize methods, such as the proximity ligation assay and SuperResolution experiments outlined in the original plan, that will inform us on the intracellular location where CALR-mediated signaling is taking place. Our preliminary data suggests there is little or no secreted CALR, so we expect this location to be the ER. The papers by Araki and Marty also suggest that the planned calcium and APEX experiments are likely to be less informative and may be omitted or minimized in the ambitious experimental plan for next year.

- In the past year, there have also been important papers shedding light on so-called "triple-negative" or sporadic mutations in MPN (reviewed by Harrison and Vannucchi in the January 21, 2016 issue of *BLOOD*). Novel, weak gain-of-function mutations in MPL and Jak2 were discovered using whole exome or next generation sequencing in these patients, as well as rare novel mutations (ie SH2B3). Thus it will be important to fully sequence Mpl, Jak2 (and CALR) for any patients enrolled in HRRC#05-435 whose mutations are not identified by standard screening at diagnosis by the clinical laboratory. If novel mutations are discovered, we will use our battery of

assays to determine if they should be classified in the gain-of-function category and if the mutation is associated with abnormal trafficking or constitutive kinase activity.

- The acquisition of additional somatic mutations during disease progression is also of keen interest. With clinical partner, Arana Yi, we have evidence for such a patient. We anticipate completing the sequencing study and plan to prepare a case report for publication.

b. **Actual or anticipated problems or delays and actions or plans to resolve them**

- Our proposal outlined plans for use of electron microscopy to evaluate the colocalization of calreticulin and MPL, as well as the overall cellular ultrastructure in megakaryocytic cells bearing mutations in these proteins. However, there was a tragic flood in the basement of the Biomedical Research Facility where the electron microscopes were housed. There was a 6-month long process to clean up the facility, evaluate the loss for insurance, and order replacement instruments. Fortunately, the facility is now up and running – with *improved* instrumentation! We will now use a brand new Hitachi HT7700 equipped with an XR16 (16 megapixel) bottom mount camera for TEM and tilt capabilities for tomography. There is also a new Zeiss Sigma 300 SEM equipped with STEM imaging capabilities, the Atlas 5 workspace, Correlative Array Tomography and a Shuttle and Find system to allow correlative imaging between the Sigma 300 and the Zeiss LSM 800 confocal Airyscan system. The tomography capabilities of the HT7700, in particular, will be applicable in the next reporting period – after a bit of a learning curve to learn the new software. Fortunately, having this equipment at UNM means that we will not have to perform 3D reconstructions at the NCMIR in San Diego, as first proposed in the grant.
- The Animal Model shared resource at the UNM Cancer Center is undergoing a major upgrade in order to meet the new demand for patient-derived xenografts. To take advantage of this opportunity, we delayed implementation of the *in vivo* experiments described in the proposal. We expect to be able to start work on establishing the UT-7 xenograft model in NSG mice, as described in Aim 1, by November 2016. Our amended animal protocol, including the change to include personnel from the Animal Core, is currently under review.

c. **Changes that had a significant impact on expenditures**

- Since we now have an electron microscope on site with tomography capabilities, we will reallocate expenses anticipated for use of scopes at the NCMIR in San Diego to biochemical and molecular biology supplies.

- Since the Cancer Center has made considerable investments to upgrade the Animal Model Shared Resource, we will reallocate the 20 percent effort estimated for Dr. Kinjo to pay for charges from the core facility. The new full-time technical lead for the Animal Core, Dr. Irina Lagutina, will inject and monitor mice and invoice us monthly. We expect that the costs will be comparable to our earlier projections – and possibly even with a cost savings, which can be applied to biochemical reagents.

d. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

- The excellent accrual to HRRC#05-435 prompted us to explore ways to speed up processing time for precious human samples. We have recently made arrangements with the UNM Human Tissue Repository and Tissue Processing Core to receive, process and store samples within hours of their acquisition by the clinical team at the UNM Cancer Center. This professional team is HIPPA compliant and their services (billed monthly) should represent an overall cost savings. This also prevents us from setting up a satellite tissue repository in order to be compliant with new federal regulations. No other research team will have access to these de-identified samples.

- As mentioned in the proposal, use of murine bone marrow-derived megakaryocytes could be an alternative strategy to use of the UT-7 cell line for study of the effect of calreticulin and Mpl mutations on cellular ultrastructure and proplatelet progression. If warranted, this study will require a minor amendment to our existing bone marrow acquisition protocol (USDA Registration #85-R-0014, protocol #14-1001103-HSC).

- Although we maintain our own small colony of triply-deficient NSG (NOD scid gamma) mice (IACUC Protocol #14-101192-B-HSC), we will now have access to mice from the breeding colony managed by the Cancer Center Animal Model core. This is another advantage of the improvements in the Animal Core, ensuring that we will always have sufficient, low-cost mice for xenograft models established by injection of UT-7 cells or primary human bone marrow-derived megakaryocyte**s**.

6. Products

a. **Publications, conference papers, and presentations**

- Dr. Cleyrat was selected for a poster presentation at the American Society for Hematology Annual Meeting (Dec 2015). (see Appendix for abstract)
- Dr. Cleyrat was selected for a platform presentation at the FASEB Science Research Conference on Genome Editing (June 2016).
- Dr. Cleyrat was selected for a platform presentation at the European Hematology Association (EHA) annual conference (June 2017).

- Manuscript submitted (in first stage of review)

  Chabot-Richards, D.S., Lynch, D.T., Gotlib, J., George, T.I., Wilson, B.S., Vasef, M.A., Arana-Yi, C.Y. and Cleyrat, C. "Leukemic Transformation of Post-Essential Thrombocythemia Myelofibrosis: A Unique Case Presenting With Double MPL and CALR Mutations" Submitted to *Leukemia (Nature Publishing Group)*.

- Manuscript published in Blood Advances (See Appendix C)

  Cédric Cleyrat, Romain Girard, Eun H. Choi, Éric Jeziorski, Thierry Lavabre-Bertrand, Sylvie Hermouet, Serge Carillo and Bridget S. Wilson, "Gene Editing-Based Functional Rescue of a Novel Mpl Mutant Associated with Congenital Amegakaryocytic Thrombocytopenia (CAMT)"

b. **Website(s) or other Internet site(s)**

See UNM Cancer Center web pages at
http://hscnews.unm.edu/news/453-000-grant-to-help-unm-researchers-study-blood-disorders
http://cancer.unm.edu/newsroom/unm-cancer-center-scientists-look-inside-cells-to-study-blood-disorders/

c. **Technologies or techniques**

- Dr. Cleyrat has established techniques for genome editing of human hematopoietic progenitor cells, including use of a multiplex double nickase approach which consists of two separate guide RNAs -- each paired either with a CFP or a RFP reporter and coupled to a mutated Cas9 enzyme. This method is reported to be more relevant to human gene modification, since it limits off-target effects.

d. **Inventions, patent applications, and/or licenses**
Not applicable.

e. **Other Products**

- Thus far, this project has allowed us to generate several cell lines of interest such as the UT-7/Tpo cells and the UT-7 cells expressing various combinations of wildtype, or mutant, mNeonGreen-tagged Mpl with TagRFP-T-tagged calreticulin. Importantly, we have also established the genome-edited UT-7/Tpo cells expressing the calreticulin mutation of type 1 (most common mutation found in MPNs). Other genome-edited cell lines will be generated during next reporting period.
- We have collected and stored samples from over 80 MPN patients. This material is annotated for patient privacy and viable cells are in cryostorage for use in the next reporting period.

7. Participants and other collaborating organizations

a. **What individuals have worked on the project?**

Name:	Bridget Wilson, PhD
Project Role:	Principal Investigator
Research Identifier (eg, ORCHID ID)	orcid.org/0000-0002-3775-4450
Nearest person month worked:	1.2
Contribution to Project	Manuscript preparation, data review, project co-direction with Cleyrat, financial oversight
Funding Support	2P50GM085273-07 (Wilson) 09/01/09-07/31/2019 NIH/NIGMS "New Mexico Center for the Spatiotemporal Modeling of Cell Signaling" This grant supports a cross-campus, inter-institutional National Center for Systems Biology. UNM, Los Alamos National Laboratory and Burnham Sanford Research Institute are participating institutions. Role: Center Director P30CA118100 (Willman) 9/01/15–08/31/20 NIH/NCI NIH/NCI UNM Cancer Center Support Grant Role: Co-Leader, Translational Cancer Biology & Signaling Program R01GM100114 (Lidke) 05/01/12-02/28/17 NIH/NIGMS "Single molecule imaging to quantify FcεRI signaling dynamics." Role: co-I R01GM114075 (Bruchez, Lidke) 4/1/2015-1/31/2019 NIH/NIGMS "Fluorogen activiating peptide-based FRET to quantify FcεRI activation mechanisms. Role: co-I F31CA192848 (Erasmus) 3/10/2015-3/28/2018 NIH/NCI "Validation of the pre-BCR signaling complex in pre-B ALL cell model by two-color single particle tracking and peptidomimetic inhibition." Role: Primary faculty mentor NIH R13AI126647 (Wilson) 71/2016-6/30/2017 NIH/NIAID This award provided support for the July 2016 FASEB summer conference on "IgE and Allergy: 50 Years and Onward." Role: Wilson was a conference organizer.

Name:	Cédric Cleyrat
Project Role:	Co-Principal Investigator
Research Identifier (eg, ORCHID ID)	0000-0002-1928-6497
Nearest person month worked:	4.8
Contribution to Project	Laboratory lead, including planning experiments, imaging, processing of patient samples and design of CRISPR genome editing protocols. Supervisor for Choi.
Funding Support	American Cancer Society-126768-IRG-14-187-19 (**PI: Cleyrat**) 04/2015-03/2017 Pilot study on myeloproliferative neoplasm diseases progression and leukemic transformation. Role: PI NIH NIGMS, P50 GM085273-06 (PI: Wilson) 8/2009-07/2019 Center for the Spatiotemporal Modeling of Cell Signaling Networks (STMC) *Role: co-Investigator (since 01/2015)*

Name:	Ichiko Kinjo
Project Role:	Senior postdoc
Research Identifier (eg, ORCHID ID)	n/a
Nearest person month worked:	2.4
Contribution to Project	Planning for xenograft model, flow cytometry. *Note that Dr. Kinjo's role in the next reporting period will be replaced by use of the Cancer Center Animal Model Core, on a fee-for-service basis.*
Funding Support	n/a

Name:	Eunice Choi
Project Role:	Technician
Research Identifier (eg, ORCHID ID)	n/a
Nearest person month worked:	7.2
Contribution to Project	Ms. Choi was recruited to the project based upon her experience with CRISPR and Talen genome editing. Under Cleyrat's supervision, she conducts all hands-on experiments for genome editing Baf3 and UT-7 cells, followed by cell sorting and testing. She also assists with processing of patient samples.
Funding Support	n/a

b. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

The following support ended during this period:

- **Wilson**, subaward PI NIH NIAID R01AI097154 (Werner) 8/1/12-31/16
  "Three-dimensional molecular tracking of IgE-FcεRI in live cells."
- **Wilson, PI,** MedImmune, LCC. Sponsored Research Agreement. 1/2012-12/2015
  "Mechanistic profiling of anti-CD22 immunotoxin for treatment of pre-B ALL"

The following support began during this period:

- Cleyrat, Cédric Center for Integrated Nanotechnology 1/2016-12/2018
  "Three-dimensional molecular tracking of EGFR in Live Renal Epithelial Cells"
- Wilson, PI P50GM085273-S1 Administrative Diversity Supplement 6/1/2016-7/31/2018

c. **What other organizations were involved as partners?**

Organization Name: Inserm UMR892/CNRS UMR6299, Centre de Recherche en Cancérologie Nantes-Angers (CRCNA), Institut de Recherche en Santé - 2 (IRS-2) - Université de Nantes
Location: Nantes, France (Sylvie Hermouet)
Partner's contribution to the project: collaboration

Organization Name: University Hospital of Nîmes, Laboratory of Clinical Cytology and Cytogenetics (Eric Carillo)
Location: Nimes, France
Partner's contribution to the project: collaboration

8. Special Reporting Requirements

a. **Collaborative awards.** *Not applicable*

9. Appendices

A. Abstract for American Society for Hematology Annual Meeting 2015.
B. Abstract for European Hematology Association Annual Conference 2017.
C. Abstract for American Society for Hematology Annual Meeting 2017.
D. Published article in Blood Advances.

APPENDIX A

Abstract for the 2015 ASH annual Meeting (Supported in part by DOD)

In Vitro Functional Rescue of a Double MPL K39N/W272R Mutant Associated with Congenital Amegakaryocytic Thrombocytopenia (CAMT) Using CRISPR/Cas9

Cédric Cleyrat[1,2], Romain Girard[1,] Éric Jeziorski[3], Thierry Lavabre-Bertrand[4], Sylvie Hermouet[2], Serge Carillo[4] and Bridget S. Wilson[1]

[1]Department of Pathology & Cancer Center, University of New Mexico Health Sciences Center, Albuquerque, NM, USA ; [2]INSERM UMR892/CNRS UMR6299, Centre de Recherche en Cancérologie Nantes-Angers (CRCNA), Institut de Recherche Thérapeutique-Université de Nantes, Nantes, France ; [3]Service de Pédiatrie III, Hôpital Arnaud de Villeneuve, Montpellier, France ; [4]University Hospital of Nîmes, Laboratory of Clinical Cytology and Cytogenetics, Nîmes, France.

Introduction: Thrombopoietin (Tpo) and its receptor, Mpl, are the principal regulators of early and late thrombopoiesis. Mutations in MPL can drastically impair its function and be a contributing factor in chronic thrombocytosis and in congenital amegakaryocytic thrombocytopenia (CAMT). CAMT is a rare inherited syndrome characterized by thrombocytopenia at birth, progressing to bone marrow failure and pancytopenia. The functional impact of CAMT mutations on Mpl signaling/trafficking is yet to be determined and could be relevant to multiple hematologic malignancies. Here we report unique familial cases of CAMT presenting with a previously unreported MPL mutation: T814C (W272R) in the background of the MPL K39N (Baltimore mutation), known to cause hereditary thrombocytosis (HT).

Patients: Consanguineous parents and their eldest daughter, all heterozygous for Mpl K39N/W272R, do not present any signs of disease. Their monozygotic twin daughters presented at birth with severe thrombocytopenia (platelet counts: 12,000/L and 14,000/L), low hemoglobin levels (10.4 mg/dL and 7.8 mg/dL) and very high Tpo levels (3,650 pg/mL and 3115 pg/mL). Bone marrow smears performed 19 days after birth showed severe megakaryocytopenia (only 1 megakaryocyte (MK) seen). Bone marrow colony formation assays yielded 3 and 9 MK colonies vs 84 MK colonies/105 mononuclear cells for the control, leading to a diagnosis of CAMT type I. Whole blood sequencing revealed the presence of a homozygous double MPL K39N/W272R mutation. One of the twins died after bone marrow transplant. A younger male sibling, homozygous for MPL K39N/W272R mutation, has also been diagnosed with CAMT type I.

Objectives: This study focuses on the functional characterization of the novel MPL W272R and K39N/W272R mutations and in vitro genetic engineering as a potential therapeutic option for CAMT.

Methods: Human megakaryoblastic UT-7 and murine Ba/F3 cells stably expressing human wild-type (WT) Mpl or K39N, W272R or doubly mutated K39N/W272R Mpl fused to mNeonGreen were used as models. Confocal microscopy, proliferation and surface biotinylation assays, as well as co-immunoprecipitation (co-IP) and western blotting analysis, were used to elucidate the function and trafficking of Mpl mutants. CRISPR/Cas9 genetic engineering was used to repair mutant MPL and rescue its function.

Results: Confocal microscopy shows that a significant fraction of chimeric WT Mpl protein reaches the cell surface. Significant surface expression is also noted for Mpl K39N. In contrast, the chimeric Mpl protein bearing the W272R mutation, alone or together with the K39N mutation, showed no detectable surface expression of the Tpo receptor. These results were confirmed by surface biotinylation assay. Co-expression of WT CALR fused to RFP, used as an ER marker, showed significantly higher co-localization (Pearson's R value) with mutant Mpl than with WT Mpl, evidence that the large majority of receptors were retained within the ER. We also evaluated Tpo signaling through the JAK/STAT, MAPK and PI3K pathways and Tpo-induced proliferation. Both WT and K39N-mutated Mpl were competent for signaling, while single or double mutants bearing W272R were unresponsive to Tpo. Tpo-induced signaling was partially restored via GRASP55 over-expression (forcing ER-trapped Mpl to traffic to the cell surface). Genetic engineering performed on cells carrying the W272R mutation restored the WT sequence and the response to Tpo, with similar cell proliferation as WT Mpl cells. In addition, co-IP studies indicate that Jak2 associates strongly with WT Mpl and Mpl K39N but not with Mpl W272R.

Conclusion: We report a new mutation of Mpl (W272R) present in cis with HT-causing K39N mutation in the context of CAMT. The absence of symptoms in the Mpl K39N/W272R-mutated parents (found to be heterozygous) can be explained by the opposite (apparently neutralizing) effects of the two mutations on the trafficking and signaling of Mpl, as shown by confocal microscopy, western blotting and proliferation assays. In children homozygous for Mpl K39N/W272R, the W272R mutation prevented Mpl binding of Jak2 and expression at cell surface, rendering Tpo signaling impossible despite the presence of the K39N mutation. Function of the deficient Mpl receptor could be rescued using two separate approaches: CRISPR/Cas9 genetic engineering and GRASP55 over-expression. Cell-permeable Tpo analogs will also be tested.

APPENDIX B

Abstract for the EHA Annual Conference (Supported in part by DOD)

Congenital Amegakaryocytic Thrombocytopenia: Functional rescue of a novel Mpl mutant in primary hematopoietic cells using CRISPR-Cas9

Cédric Cleyrat[1,2], Romain Girard[1], Éric Jeziorski[3], Thierry Lavabre-Bertrand[4], Sylvie Hermouet[2], Serge Carillo[4] and Bridget S. Wilson[1]

[1]Department of Pathology & Cancer Center, University of New Mexico Health Sciences Center, Albuquerque, NM, USA ; [2]INSERM UMR892/CNRS UMR6299, Centre de Recherche en Cancérologie Nantes-Angers (CRCNA), Institut de Recherche Thérapeutique-Université de Nantes, Nantes, France ; [3]Service de Pédiatrie III, Hôpital Arnaud de Villeneuve, Montpellier, France ; [4]University Hospital of Nîmes, Laboratory of Clinical Cytology and Cytogenetics, Nîmes, France.

Keywords: Thrombocytopenia, c-Mpl, Gene Therapy, HSPCs

Background: Thrombopoietin (Tpo) and its receptor, Mpl, are the principal regulators of early/late thrombopoiesis and hematopoietic stem cells maintenance. Mutations in *MPL* can drastically impair its function and be a contributing factor in multiple hematologic malignancies, including congenital amegakaryocytic thrombocytopenia (CAMT). CAMT is a rare inherited syndrome characterized by thrombocytopenia at birth, progressing to bone marrow failure and pancytopenia. The functional impact of CAMT mutations on Mpl is yet to be determined. Here we report unique familial cases of CAMT presenting with a previously unreported *MPL* mutation: T814C (W272R) in the background of the activating *MPL* G117T (K39N or Baltimore) mutation.

Patients: Consanguineous parents and their eldest daughter, all heterozygous for Mpl K39N/W272R, do not present any signs of disease. Their monozygotic twin daughters presented at birth with severe thrombocytopenia leading to a diagnosis of CAMT type I. Whole blood sequencing revealed the presence of a homozygous double Mpl K39N/W272R mutation, as their younger male sibling. One of the twins died after bone marrow transplant.

Aim: This study focuses on the functional characterization of this novel Mpl mutant and the use of genome editing as a novel therapeutic option for CAMT.

Methods: Human megakaryoblastic UT-7 and murine Ba/F3 cells stably expressing human wild-type (WT) Mpl or mutant Mpl fused to mNeonGreen were used as models. Confocal microscopy, proliferation and surface biotinylation assays, as well as co-immunoprecipitation and western blotting analysis, were used to elucidate the function and trafficking of Mpl mutants. Multiplex, flow-based, CRISPR-Cas9 gene editing was used to repair mutant *MPL* and rescue its function. Cord blood from the younger male sibling was used as a source of primary homozygous Mpl K39N/W272R $CD34^+$ cells. $CD34^+$ cells were edited using ribonucleoproteins electroporation followed by sequencing and functional assays such as flow cytometry and single colony assays.

Results: Confocal microscopy shows that a significant fraction of chimeric WT Mpl protein reaches the cell surface. Significant surface expression is also noted for Mpl K39N. In contrast, the chimeric Mpl protein bearing the W272R mutation, alone or together with the K39N mutation, showed no detectable surface expression of the Tpo receptor while being strongly co-localized with ER marker calreticulin. Both WT and K39N-mutated Mpl were found signaling competent, while single or double mutants bearing W272R were unresponsive to Tpo. Tpo-induced signaling was partially restored via GRASP55 over-expression (forcing ER-trapped Mpl to traffic to the cell surface). Genome editing performed on cells carrying the W272R mutation restored the WT sequence and the response to Tpo, with similar cell proliferation as WT Mpl cells. Finally, when applied to primary Mpl K39N/W272R $CD34^+$ cells, CRISPR-based gene editing rescued surface expression of Mpl and response to Tpo, as assessed by flow cytometry. Furthermore, edited $CD34^+$ cells were able to generate a similar number of megakaryocytic colonies as control $CD34^+$ cells in a single colony assay. Non-edited cells failed to do so.

Conclusion: We report a new double *in cis* mutation of Mpl (K39N/W272R) in the context of CAMT. Function of the deficient Mpl receptor could be rescued using two separate approaches: GRASP55 over-expression and CRISPR-Cas9 genome engineering. Successful editing of primary hematopoietic stem cells indicates direct therapeutic applications for gene editing in this disease.

APPENDIX C

Abstract for the 2017 ASH annual Meeting (Supported in part by DOD)

Leukemic Transformation of Post-Essential Thrombocythemia Myelofibrosis: A Unique Case Presenting With Double *MPL* and *CALR* Mutations

Cédric Cleyrat[1,2], Devon S. Chabot-Richards[1*], David T. Lynch[1,3*], Jason Gotlib[4], Tracy I. George[1], Bridget S. Wilson[1,2], Mohammad A. Vasef[1] and Cecilia Y. Arana-Yi[2]

[1]Department of Pathology & [2]Comprehensive Cancer Center, University of New Mexico Health Sciences Center, Albuquerque, NM, USA; [3]San Antonio Military Medical Center, Fort Sam Houston, San Antonio, TX, USA; [4]Department of Medicine/Hematology & Stanford Cancer Institute, Stanford University School of Medicine, Stanford, CA, USA. *Both authors contributed equally to this work

Keywords. Myeloproliferative Neoplasms, Essential Thrombocythemia, Myelofibrosis, c-Mpl, Calreticulin, Acute Myeloid Leukemia.

Background. Activating mutations in the thrombopoietin receptor (*MPL*), its proximal signaling partner Janus Kinase 2 (*JAK2*) or its endoplasmic reticulum chaperone, calreticulin (*CALR*), can lead to myeloproliferative neoplasms (MPNs). These chronic hematopoietic clonal neoplasms are associated with abnormal myeloid cell proliferation, cytokine production, as well as bone marrow fibrosis. The precise pathogenic mechanism leading to essential thrombocythemia (ET) and primary myelofibrosis (PMF), two closely linked MPNs, and the functional impact of *MPL* and *CALR* mutations on Mpl signaling and intracellular trafficking are yet to be determined. MPNs can evolve into acute myeloid leukemia (AML); however, the mechanisms of this transformation are mostly unknown. Here we report a unique case of post-ET MF with AML transformation, presenting with previously unreported double mutations: *MPL*W515R and a *CALR* 52-base pair deletion (Type I).

Case Report. A 69 yo female was diagnosed with ET in 1996, and subsequently progressed to post-ET MF in 2008. Molecular studies in 2008 were negative for *JAK2*V617F and the *BCR*/*ABL1* fusion. At this time, the patient was treated with hydroxyurea and erythropoietin. In 2010, the patient participated in the phase III COMFORT-I trial of ruxolitinib versus placebo and was randomized to ruxolitinib. Ruxolitinib was discontinued in 2014 due to severe anemia and transfusion dependence. The patient also showed no response after 3 cycles of the phase I/II trial of PRI-724, a β-catenin/wnt pathway inhibitor. She resumed ruxolitinib in 2015, but developed worsening leukocytosis and myeloid immaturity, progressing to AML in January 2016. She stopped ruxolitinib and received azacytidine for one cycle. However, the patient developed tumor lysis syndrome and acute on chronic kidney injury and expired. Molecular studies at the time of leukemic transformation revealed *CALR* type I and *MPL* W515R mutations. A repeat test for *JAK2*V617F was negative.

Aim. This study focuses on the functional characterization of the *MPL*/*CALR* double mutations, their impact on Mpl intracellular trafficking and signaling, and their correlation with disease progression.

Methods. Cryopreserved PBMCs and/or granulocytes obtained at diagnosis and upon transformation to AML were used as source of primary cells to determine the mutational status of the patient. Sequencing, single colony assay (SCA), proximity ligation assay, flow cytometry, biotinylation assays, as well as western blotting analysis, performed on primary cells, were used to elucidate the function and trafficking of Mpl mutants in the context of mutant calreticulin. Multiplex cytokine profiling at different stages of disease progression was also evaluated. UT-7, megakaryoblastic cells, were subjected to CRISPR/Cas9-based genome engineering to model Mpl and calreticulin mutations and further investigate the complex interplay between these players in MPNs pathogenesis.

Results. At diagnosis, Sanger sequencing revealed the presence of a *CALR* Type I mutation while *JAK2* and *MPL* were negative. gDNA sequencing of samples collected between diagnosis and AML transformation showed the presence of consistent heterozygous *CALR* Type I mutation but expanding *MPL*W515R mutation. Analysis of ~300 single colonies at transformation, grown in the presence (200 colonies) or in the absence (100 colonies) of cytokines, confirmed the presence of heterozygous *CALR* mutations in 100% of tested colonies. Notably, 22.4% were WT, 75.3% were heterozygous and 2.3% were homozygous for *MPL*W515R. Additionally, a myeloid gene panel analysis at AML transformation showed the presence of an additional *ASXL1* mutation. Proximity ligation/microscopy assay performed on $CD34^+$ cells showed that Mpl interacts with mutant calreticulin in a different cellular compartment, outside the ER and near the plasma membrane, than with wildtype calreticulin, indicating that receptor trafficking and signaling are closely related in MPN pathogenesis.

Conclusion. We report a unique case of *CALR* mutation associated with post-ET MF. Subsequent acquisition of *MPL*W515R by the MPN clone occurred temporally with AML transformation. Mechanistic studies using gene-edited UT-7 cell models are ongoing. This study will provide important insight in the understanding of MPNs progression and the relative contribution of *CALR* and *MPL* mutations to neoplastic transformation.

APPENDIX D

Published article in Blood Advances.

http://www.bloodadvances.org/content/1/21/1815

REGULAR ARTICLE

blood advances

Gene editing rescue of a novel *MPL* mutant associated with congenital amegakaryocytic thrombocytopenia

Cédric Cleyrat,[1] Romain Girard,[1] Eun H. Choi,[1] Éric Jeziorski,[2] Thierry Lavabre-Bertrand,[3] Sylvie Hermouet,[4] Serge Carillo,[3,*] and Bridget S. Wilson[1,*]

[1]Department of Pathology and Comprehensive Cancer Center, University of New Mexico Health Sciences Center, Albuquerque, NM; [2]Service de Pédiatrie III, Hôpital Arnaud de Villeneuve, Montpellier, France; [3]University Hospital of Nîmes, Laboratory of Clinical Cytology and Cytogenetics, Nîmes, France; and [4]Centre de Recherche en Cancérologie et Immunologie Nantes-Angers, INSERM U1232, Institut de Recherche en Santé 2–Biotech, Nantes, France

Key Points

- We report unique familial cases of CAMT presenting with a novel *MPL* W272R mutation in the background of the activating *MPL* K39N mutation.
- Function of mutant *Mpl* receptor can be rescued using 2 approaches: autophagic cell surface delivery and CRISPR-Cas9 gene editing.

Thrombopoietin (Tpo) and its receptor (Mpl) are the principal regulators of early and late thrombopoiesis and hematopoietic stem cell maintenance. Mutations in *MPL* can drastically impair its function and be a contributing factor in multiple hematologic malignancies, including congenital amegakaryocytic thrombocytopenia (CAMT). CAMT is characterized by severe thrombocytopenia at birth, which progresses to bone marrow failure and pancytopenia. Here we report unique familial cases of CAMT that presented with a previously unreported *MPL* mutation: T814C (W272R) in the background of the activating *MPL* G117T (K39N or Baltimore) mutation. Confocal microscopy, proliferation and surface biotinylation assays, co-immunoprecipitation, and western blotting analysis were used to elucidate the function and trafficking of Mpl mutants. Results showed that Mpl protein bearing the W272R mutation, alone or together with the K39N mutation, lacks detectable surface expression while being strongly colocalized with the endoplasmic reticulum (ER) marker calreticulin. Both WT and K39N-mutated Mpl were found to be signaling competent, but single or double mutants bearing W272R were unresponsive to Tpo. Function of the deficient Mpl receptor could be rescued by using 2 separate approaches: (1) GRASP55 overexpression, which partially restored Tpo-induced signaling of mutant Mpl by activating an autophagy-dependent secretory pathway and thus forcing ER-trapped immature receptors to traffic to the cell surface; and (2) CRISPR-Cas9 gene editing used to repair *MPL* T814C mutation in transfected cell lines and primary umbilical cord blood–derived $CD34^+$ cells. We demonstrate proof of principle for rescue of mutant Mpl function by using gene editing of primary hematopoietic stem cells, which indicates direct therapeutic applications for CAMT patients.

Introduction

Thrombopoietin (Tpo) and its receptor (Mpl) are the principal regulators of early and late thrombopoiesis and hematopoietic stem cell (HSC) maintenance. Germline or somatic mutations in *MPL* are thus contributing factors in multiple hematopoietic diseases. Gain-of-function mutations in *MPL* are associated with myeloproliferative neoplasms (essential thrombocythemia, primary myelofibrosis) and hereditary thrombocytosis, whereas loss-of-function mutations can be directly linked to bone marrow failure syndromes such as congenital amegakaryocytic thrombocytopenia (CAMT). CAMT is a rare inherited syndrome characterized by thrombocytopenia at birth that rapidly progresses to bone marrow failure and pancytopenia. Since the first description of a disease-associated *MPL* mutation in CAMT in

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*S.C. and B.S.W. contributed equally to this study.

The full-text version of this article contains a data supplement.

1999,[1] more than 50 different genetic events have been reported for *MPL*,[2,3] and they are sometimes associated with defects in surface presentation.[4-6] Notably, the *MPL* Baltimore substitution (K39N) is associated with high platelet counts in patients of African American descent, despite incomplete processing and reduced Mpl protein levels.[7]

Although cell surface expression of Mpl is required for stimulation by its ligand (Tpo), complex relationships between mutant and wild-type (WT) forms of Mpl, Jak2, and the endoplasmic reticulum (ER) chaperone calreticulin govern both the intracellular trafficking of receptors and signal propagation.[8-10] Prior work has shown that the canonical ER-Golgi route for trafficking of Mpl to the cell surface is aberrant in myeloproliferative neoplasms, linked in part to requirements for WT Jak2 acting as a chaperone.[11,12] An alternative pathway to the surface for Mpl is provided by an unconventional autophagy-linked secretory pathway.[13] It is important to determine the functional impact of CAMT mutations on Mpl signaling and trafficking, because clinical presentation, disease progression, and treatment options reflect the underlying cellular mechanisms.[6,14,15]

In this study, the severity of CAMT type I disease in 3 siblings was associated with a homozygous double *MPL* K39N/W272R mutant that resulted in complete blocking of Mpl trafficking to the plasma membrane. Currently, HSC transplantation is the only curative option for pediatric patients with life-threatening CAMT.[15,16] We show that CRISPR-Cas9 gene editing methods[17] could be used to correct abnormalities in the *MPL* gene.

Methods

Patient and healthy donor material

All material from healthy donors (HDs) and patients was obtained after written informed consent, according to institutional guidelines.

Culture of primary cells and cell lines

Murine Ba/F3 and human UT-7 cells were obtained from DSMZ (Braunschweig, Germany) and maintained according to the supplier's recommendations. $CD34^+$ cells were maintained and expanded in StemSpan SFEM-II media (STEMCELL Technologies) supplemented with thrombopoietin, interleukin-6 (IL-6), Flt-3, and stem cell factor, all at 100 ng/mL (Peprotech).

MPL complementary DNA subcloning and sequencing

Messenger RNA molecules were purified from cord blood (CB) cells isolated from patient II.4. Reverse transcription was performed by using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific), followed by primer-specific complementary DNA (cDNA) amplification, subcloning, and sequencing (for primers sequences, see supplemental Table 1).

cDNA constructs and transfection

Human *MPL* cDNA fused to mNeonGreen was generated by gene fusion polymerase chain reaction (PCR) using Kapa HiFi Hotstart DNA Polymerase (Kapa Biosystems) and was cloned into pcDNA3.1 (Life Technologies). K39N and W272R mutations were inserted by site-directed mutagenesis (for primer sequences, see supplemental Table 1). The same cloning technique was used to generate the TagRFP-T–tagged human calreticulin cDNA (Sino Biological) into pcDNA6.2. All constructs were checked by sequencing. Transient transfections of Ba/F3 and UT-7 cells were performed by using the Amaxa Nucleofector device (Lonza). Cells (10×10^6) were transfected with 25 μg of plasmid DNA following the supplier's recommendations. Cells were harvested for experiments 24 hours later. To generate stably expressing Mpl-mNeonGreen (Mpl^{mNG}) variants, cells were selected by using geneticin and fluorescence-activated cell sorting.

Immunoblots

Cells (10×10^6) were harvested and treated, or not, with recombinant human Tpo (PeproTech) at 50 ng/mL for 10 minutes at 37°C, then lysed in radioimmunoprecipitation assay (RIPA) buffer. Then, 25 μg of total proteins was loaded on 4% to 15% polyacrylamide gradient gels (Bio-Rad) and transferred to nitrocellulose membranes (iBlot 2 device, Life Technologies). After blocking, membranes were probed with primary antibodies for Mpl or β-actin (Millipore), phosphorylated (p)-Jak2, p-Stat1, pStat5, p-Akt, p-Erk1/2, calreticulin (CRT), or calnexin (CANX) (Cell Signaling Technology). In some cases, membranes were stripped once (Thermo Fisher Scientific) and then re-probed. After incubation with horseradish peroxidase–conjugated secondary antibodies, reactive bands were revealed with a chemiluminescent substrate (Thermo Fisher Scientific) and imaged with a Bio-Rad ChemiDoc XRS+ imaging system (Bio-Rad) equipped with Image Lab 4.0.1 software.

Co-immunoprecipitation assay

Cells (25×10^6) were lysed in 1 mL of RIPA buffer (without vortexing) for 20 minutes on ice, then spun down at 13 000*g* for 20 minutes at 4°C. mNeonGreen-tagged Mpl proteins were immunoprecipitated by using mNeonGreen-nAb–conjugated agarose beads (Allele Biotechnology), following the manufacturer's instructions, before denaturation in Laemmli buffer and western blot analysis.

Biotinylation assay

For each condition, 25×10^6 parental or transfected UT-7 cells were harvested, washed 3 times in ice-cold phosphate-buffered saline (PBS), and re-suspended in 1 mL of PBS containing 1.5 mg of Sulfo-NHS-Biotin (Thermo Fisher Scientific). Biotinylation reactions proceeded for 30 minutes on ice, followed by 3 ice-cold washes in PBS supplemented with 100 mM glycine. Cells were lysed in RIPA buffer, and biotinylated proteins were immunoprecipitated by using streptavidin-agarose beads (Life Technologies). Samples were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting.

Confocal imaging and processing

Confocal images were acquired on a Zeiss LSM 510 META microscope equipped with a 405-nm laser diode, argon and helium-neon 543-nm lasers, a 63× differential interference contrast oil or water objective and ZEN software.

Statistical analysis

Statistical analysis was performed and diagrams were made by using GraphPad Prism 5. Statistical tests are described in the figure legends. Pearson's pixel intensity correlation over space coefficient, *R*, of 2D bicolor confocal images was calculated by using the FIJI Coloc_2 plugin.

Genome editing

For editing Ba/F3 cells, a high-scoring guide RNA (gRNA) (gRNA#1WR) was designed by using the CRISPR portal (http://crispr.mit.edu/) and then subcloned into a modified PX458 plasmid (Addgene #48138). For subcloning, 2 partially complementary oligonucleotides (Integrated DNA Technologies) were assembled by PCR. Gel-purified PCR products were cloned into a BbsI-digested

PX458 plasmid by using Gibson Assembly (New England Biolabs). After sequencing, the final plasmid with the repair template single-stranded oligo donor DNA (ssODN) W272R>WT #1 was used to electroporate Ba/F3 cells expressing Mpl W272R mutant. Red fluorescent protein (RFP)–expressing cells were selected by flow cytometry and transferred to an IL-3–deprived/Tpo-supplemented media before performing a cell proliferation assay. Editing of UT-7 cells expressing Mpl W272R mutant was conducted by using a double nickase approach. Two complementary gRNAs (#2 and #3) were designed and built similarly to gRNA #1WR. gRNAs #2 and #3 were subcloned into PX461 vectors (Addgene #48140), modified in-house for expression of a cyan fluorescent protein (CFP) or RFP reporter. UT-7 cells were transfected with both gRNAs and the repair template ssODN W272R>WT #2. Doubly transfected cells were flow sorted and plated in the absence of granulocyte-macrophage colony-stimulating factor and in the presence of Tpo before proceeding with a cell proliferation assay. For K562 and primary $CD34^+$ cell editing, a WT-specific version of gRNA #1WR was built (gRNA #1WT) and was subcloned as described before. gRNAs #1WT and #1WR were also produced as in vitro transcribed RNA and then complexed with Sp. Cas9 to form ribonucleoprotein (RNP) complexes as described.[18] See supplemental Table 1 for gRNAs, primers, and repair template sequences.

In vitro cutting assay

Sp. Cas9 protein (50 ng; New England Biolabs) was mixed with 250 ng of in vitro–transcribed gRNA (either #1WT or #1WR) and incubated at 37°C for 5 minutes in a PCR device to form RNPs. RNPs were then combined with 150-ng target templates, incubated for 1 hour at 37°C, and then denatured for 5 minutes at 80°C. Reaction products were loaded on a 1.2% agarose gel, stained with GelGreen, and quantified with a Bio-Rad ChemiDoc XRS+ imaging system (Bio-Rad) equipped with Image Lab 4.0.1 software.

Megakaryocytic colony assay

Colony assays were conducted as described.[19] Starting materials were CB-derived $CD34^+$ cells from HDs (StemExpress) or study patient II.4, with or without editing.

Flow cytometry

Five days after gene editing, control, II.4, and II.4-edited $CD34^+$ cells were harvested and incubated with human Fc block. Cells were then incubated with an anti-Mpl (CD110)-AlexaFluor 647 dye or an isotype control also coupled to AlexaFluor 647 following the manufacturer's recommendations. Fc block reagent and antibodies were from BD Biosciences. Data were acquired on a Becton Dickinson LSRFortessa flow cytometer.

Proliferation assay

Parental, stably transfected, or CRISPR-edited cells (Ba/F3 or UT-7) were plated in 96-well plates at 5000 cells per well and incubated for 0 to 120 hours with 10 ng/mL Tpo. Cell proliferation was assessed by using an XTT II colorimetric assay (Roche) following the manufacturer's recommendations.

Amplicon sequencing by next-generation sequencing

gDNA purified from gene-edited $CD34^+$ cells from patient II.4 was used as a PCR template using *MPL* exon 5 sequencing primers (for sequences, see supplemental Table 1). Purified PCR products were sequenced using next-generation amplicon sequencing by Genewiz Inc., and results were analyzed by using Integrative Genomics Viewer software (http://software.broadinstitute.org/software/igv).

Results

Study family

In a French family of Moroccan descent, consanguineous parents (first-degree cousins) and their eldest daughter were found to be heterozygous for 2 *MPL* mutations: the activating K39N mutation and a previously unreported W272R mutation of unknown function. These 3 individuals had normal platelet counts and were asymptomatic (Figure 1A). Monozygotic twin daughters presented at birth with severe thrombocytopenia (platelet counts: 12 000/L and 14 000/L), low hemoglobin levels (104 g/L and 78 g/L), and very high Tpo levels (3650 pg/mL and 3115 pg/mL) but normal white blood cell counts (13.4×10^9/L and 9.7×10^9/L). Bone marrow smears performed 19 days after birth showed only a single megakaryocyte, indicative of severe megakaryocytopenia. Bone marrow colony formation assays yielded 3 and 9 megakaryocytic colonies vs 84 megakaryocytic colonies per 10^5 mononuclear cells for the control, leading to a diagnosis of CAMT type I. Whole-blood sequencing revealed the presence of homozygous *MPL* K39N/W272R mutations. The twins underwent bone marrow transplantation and 1 survived. A younger male sibling was subsequently found to be homozygous for the *MPL* K39N/W272R mutations. He was diagnosed with CAMT type I and received bone marrow transplantation. Additional follow-up was not possible because the family relocated.

Novel CAMT-causing W272R *MPL* mutation is associated in cis with *MPL* K39N

Sequencing of genomic DNA isolated from peripheral blood from both parents and their 4 children showed the presence of the K39N (G117T) polymorphism in exon 2, along with an exon 5 mutation at W272R (T814C) (Figure 1A). Sequencing of cDNA, generated by reverse transcription PCR of CB-derived messenger RNA, revealed that mutations were associated in cis (13 of 13 cDNA clones). The asymptomatic parents and 1 sibling were heterozygous, whereas the 3 symptomatic children were homozygous for the double *MPL* mutation. The schematic in Figure 1B shows that both amino acid substitutions are located in the Mpl extracellular domain. The W272R mutation lies within the conserved WGSWS motif.[20]

Mpl mutants do not respond to ligand stimulation

Functional effects of Mpl mutations were investigated in murine Ba/F3 cells and in human megakaryoblastic UT-7 cells. Ba/F3 cells are an IL-3–dependent cell model; UT-7 cells depend on granulocyte-macrophage colony-stimulating factor.[21] The relative contributions of the variants upon Mpl trafficking were revealed in live cells expressing Mpl-mNeonGreen fusion proteins (Mpl^{mNG}) as WT, single substitutions, or double substitutions in both cell lines. Western blotting results in Ba/F3 cells (Figure 2A) showed that the WT and K39N forms of Mpl are signaling competent, based upon elevated levels of phosphorylated signaling proteins after stimulation of cells with Tpo. In contrast, cells expressing Mpl bearing either the single W272R or the double K39N/W272R mutation did not respond to ligand stimulation. Note that WT Mpl migration during gel electrophoresis resolved the receptor into 2 different forms: the upper band represents mature glycosylated Mpl (indicating passage through the Golgi), and the lower band represents core-glycosylated Mpl (ER pattern).[13] Both forms were seen in cells expressing WT or K39N Mpl, but only the lower form was seen for Mpl mutants bearing the W272R mutation, indicating failure to progress from the ER to the Golgi.

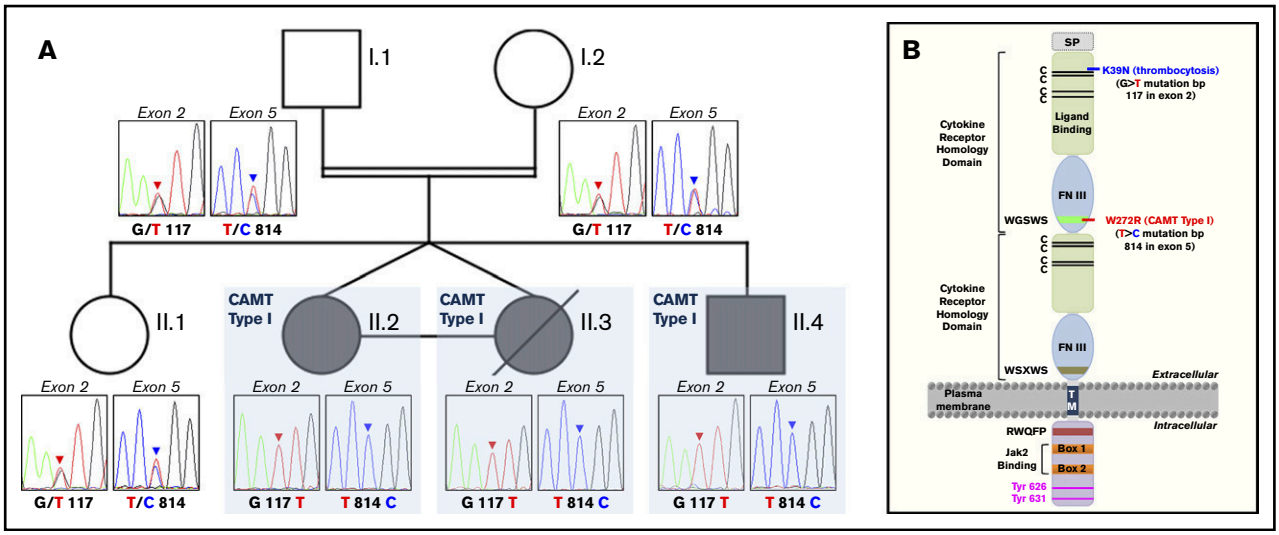

Figure 1. Novel in cis double *MPL* mutation is associated with familial CAMT type I. (A) Pedigree tree that illustrates the autosomal recessive transmission pattern in this family. Circles represent females and squares represent males. Open symbols indicate healthy family members, filled symbols indicate family members with CAMT type I, single horizontal line connecting 2 symbols indicates monozygotic twins, and slashes represent deceased family members. The genotypes of all family members are presented as genomic DNA sequencing chromatograms. Family members I.1, I.2, and II.1 are heterozygous for the 2 G117T mutation in exon 2 and the T814C mutation in exon 5. Family members II.2, II.3, and II.4 are homozygous for both mutations. (B) Schematic representation of the functional domains of the Tpo receptor and the location of extracellular G117T (K39N) polymorphism and T814C (W272R) mutation. C, cysteine residue; FN III, fibronectin III domain; SP, signal peptide; TM, transmembrane domain; Tyr, tyrosine residue.

Mpl W272R and K39N/W272R are retained in the ER

Confocal microscopy studies (Figure 2B) confirmed that only WT Mpl^{mNG} and Mpl K39N^{mNG} reached the cell surface in transfected Ba/F3 cells (solid arrowheads). In contrast, the chimeric Mpl^{mNG} proteins bearing the W272R mutation, alone or together with the K39N mutation, showed no detectable surface expression of the Tpo receptor. Open arrowheads (Figure 2B, lower panels) indicate the absence of a clearly labeled plasma membrane in these cells. Instead, we observed a reticular distribution of W272R and K39N/W272R mutant Mpl that is consistent with retention in the ER. After biotinylation of surface proteins, anti-biotin immunoprecipitation confirmed the absence of Mpl at the surface of cells expressing W272R mutants (Figure 2D, center panel). Further proof of the trafficking defect of mutant Mpl was obtained by co-expressing WT and mutant Mpl^{mNG} in UT-7 cells, together with WT calreticulin fused to RFP as an ER marker (Figure 2C). We applied spatial statistics methods (Pearson's *R* value) to show a significantly higher colocalization of mutant Mpl W272R^{mNG} proteins with the ER marker compared with either WT or K39N receptors. A statistically significant difference in colocalization of Mpl K39N^{mNG} with the ER compared with WT Mpl^{mNG} was also noted. Finally, results show increased co-precipitation of the mutant Mpl's with the ER resident chaperones CRT and CANX, particularly those receptors with a single mutation at W272R (Figure 2D).

Autophagy-based functional rescue of mutant Mpl surface expression

In a previous study that focused on the complex intracellular trafficking pathways of Mpl, we discovered that immature, core-glycosylated Mpl proteins were able to traffic from the ER to the plasma membrane by using an autophagy-based secretory pathway.[13] This unconventional route bypasses the canonical ER-to-Golgi-to-plasma membrane delivery route for glycosylated proteins and can be promoted in cells by overexpressing Golgi reassembly stacking protein 55 (GRASP55). Because autophagy provides an alternative pathway for misfolded proteins to reach the cell surface,[22] we tested the hypothesis that overexpression of GRASP55 might partially restore Tpo-induced signaling in cells expressing the doubly substituted Mpl K39N/W272R. Figure 3A shows results in UT-7 cells stably expressing either WT or mutant Mpl, followed by transient overexpression of V5-tagged GRASP55. GRASP55 overexpression overrode the block in signaling for the double Mpl mutant, resulting in phosphorylation of Jak2, STAT1, AKT, and Erk after Tpo challenge. Levels of stimulation were comparable to that of cells expressing WT Mpl. Results show that the doubly mutated, core-glycosylated Mpl can bind ligand and initiate signaling if it is forced to the cell surface through the autophagic secretory pathway.

Tpo analog eltrombopag fails to promote proliferation of mutant Mpl-bearing cells

The ability of UT-7 and Ba/F3 cell lines to proliferate in the presence of Tpo (10 ng/mL) was evaluated (Figure 3B-C). UT-7 and Ba/F3 cells expressing gain-of-function Mpl K39N^{mNG} (red filled squares) showed a significant increase in proliferation compared with WT Mpl^{mNG}- expressing cells (green filled diamonds). Consistent with results in Figure 2A, cells expressing Mpl W272R^{mNG} or Mpl K39N/W272R^{mNG} remained unresponsive to Tpo (cyan and purple filled triangles, respectively). Data in Figure 3C show that Ba/F3 cells expressing WT and K39N Mpl displayed similar growth curves when treated with the membrane-impermeable Tpo analog eltrombopag (dotted lines) compared with Tpo (solid lines). Ba/F3 cells expressing the W272R mutants were unresponsive to eltrombopag.

Genome engineering restores Tpo response in *MPL* W272R–transfected cell lines

CRISPR-Cas9 engineering methods were used to correct the W272R mutation in transfected cells and restore the WT sequence. As an initial proof of principle, expression plasmids in

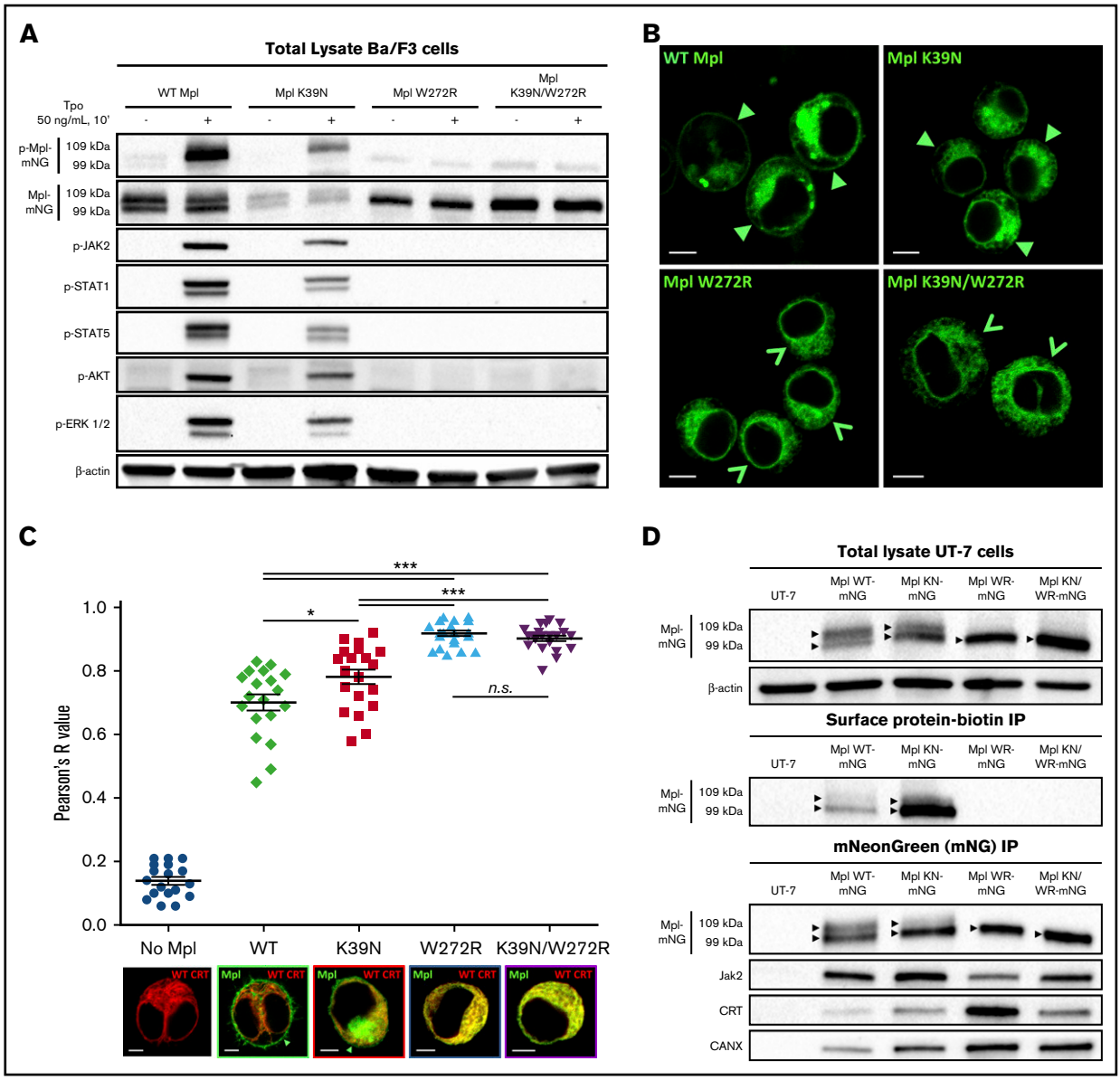

Figure 2. Mpl W272R and K39N/W272R are absent from the cell surface and do not respond to ligand stimulation. (A) Western blot results for total Mpl protein and phosphorylated (p) signaling partners in cell lysates prepared from transfected Ba/F3 cells with and without Tpo stimulation (50 ng/mL, 10 minutes, 37°C). Labels at the top indicate WT or mutant Mpl^{mNG} constructs for each cell line tested. (B) Representative confocal images of the live Ba/F3 cells used for western blot characterization in panel A. Closed arrowhead symbols indicate the presence of surface Mpl in cells expressing WT or K39N Mpl. Open arrowhead symbols point to the absence of a clearly defined plasma membrane outline in cells expressing W272R or K39N/W272R mutant Mpl. (C) Image analysis of human UT-7 cells co-expressing Mpl^{mNG} WT or mutant proteins and the ER-resident protein calreticulin (CRT) fused to TagRFP-T ($CRT^{TagRFP\text{-}T}$). Co-localization of both fluorescent markers was assessed by using Pearson's analysis of dual-channel confocal images from at least 20 cells for each condition. Means ± standard error of the mean are shown, and pairwise statistical analyses using unpaired Student *t* test are represented by horizontal bars. Representative images of each cell population are shown at the bottom of the panel. (D) Co-immunoprecipitation (IP) of mutant Mpl proteins with ER-resident proteins CRT and calnexin (CANX) in stably transfected UT-7 cells. Upper bands in the WT and K39N (KN) lanes represent fully glycosylated receptors, indicative of maturation in the Golgi. Scale bars = 5 μm. $^*P < .05$; $^{***}P < .0001$. n.s., not significant.

Ba/F3 cells were edited by using a unique single gRNA coupled to the WT Cas9 enzyme and an RFP reporter. Expression plasmids in UT-7 cells were edited by using a multiplex double nickase approach, which consists of 2 separate single gRNAs, each paired with either a CFP or an RFP reporter and coupled to a mutated Cas9 enzyme (Cas9 with the D10A mutation). For both editing strategies, an asymmetric ssODN was used as a repair template to maximize homology-directed (HDR) editing.[23] Figure 3 shows results for the restoration of growth competence to Tpo in edited cells (labeled Mpl W272R>WT; blue open circles). Figure 3B shows data for UT-7 cells, and Figure 3C shows data for Ba/F3 cells. Successfully edited cells recovered growth competence in the presence of Tpo, with growth rates similar to those of WT Mpl^{mNG} cells (green filled diamonds; Figure 3C-D).

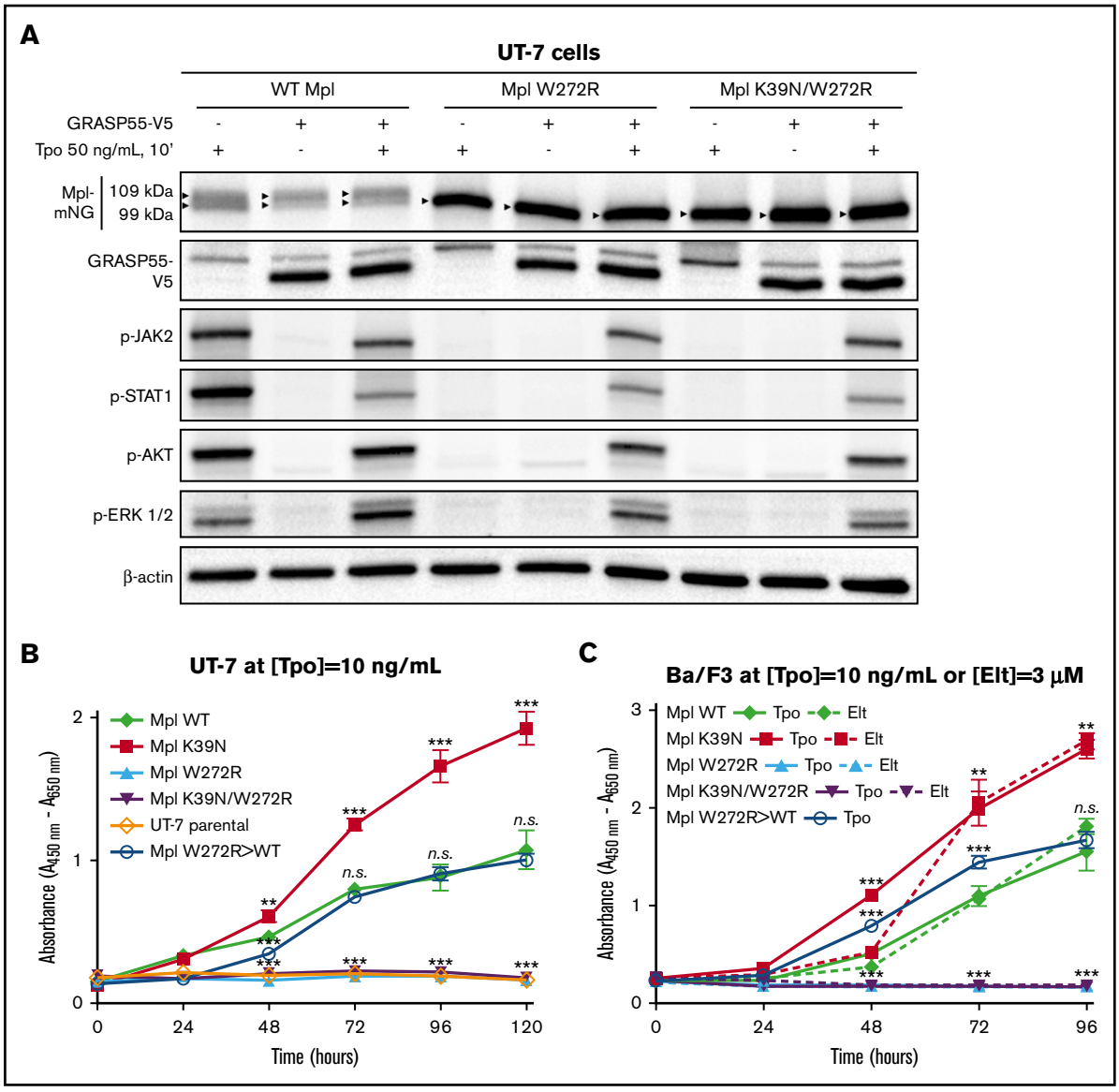

Figure 3. Gene editing or autophagic delivery of mutant Mpl to the cell surface rescue receptor function in vitro. (A) Transient overexpression of GRASP55 tagged with a V5 epitope results in accumulation of the lower-molecular-weight core-glycosylated form of Mpl regardless of WT or mutant status. Receptors are shown to be signaling competent on the basis of phosphorylation of key signaling proteins in the Jak/STAT and PI3K pathways in response to Tpo. (B-C) XTT-II proliferation assays performed on UT-7 or Ba/F3 cell lines expressing WT or mutant Mpl^{mNG} and selected for growth in the presence of Tpo (panel C, solid lines) or eltrombopag (Elt) (panel C, dotted lines). CRISPR-Cas9–edited cells that were reverse-engineered to restore the WT sequences in *MPL* exon 5 from the mutated W272R sequence (labeled Mpl W272R>WT) are represented by blue open circles. UT-7 cells were edited by using the D10A Cas9 mutant and 2 single gRNAs in a double nickase approach. A classical WT Cas9 approach (ie, coupled to a unique single gRNA) was used to edit Ba/F3 cells. $^{**}P < .005$; $^{***}P < .0001$.

Efficient editing of the *MPL* locus in K562 and CD34$^+$ cells

To test our gene editing tools, we next applied our gRNA/WT Cas9 editing approach to human K562 cells and primary HSCs. Our goals were to confirm targeting of the *MPL* locus and to demonstrate the potential to restore megakaryocytic progenitor capabilities in CAMT patient–derived CD34$^+$ cells.

Editing strategies in K562 cells

Because human K562 cells are homozygous for WT *MPL*, we designed a WT version of gRNA#1WR (named gRNA#1WT). Both gRNAs differ by only 1 bp corresponding to the T814C mutation on *MPL* that is spanned by the gRNAs, thus creating a WT-specific and a mutant (WR)-specific gRNA. The schematic in Figure 4A represents gRNA#1WT and gRNA#1WR, the W272R>WT repair template, and the W272R mutated genomic *MPL* sequence. Cas9 WT, the proto-adjacent motif (PAM), and cleavage sites are also represented. Although they are still difficult to predict, single bp mismatches located 1 to 12 bp in the PAM-proximal region of a gRNA can drastically impact its specificity.[24] To test the specificity of gRNA#1WT and gRNA#1WR, we conducted an in vitro cutting assay using WT or WR templates. Figure 4B shows that both gRNAs in the presence of their respective templates exert a very

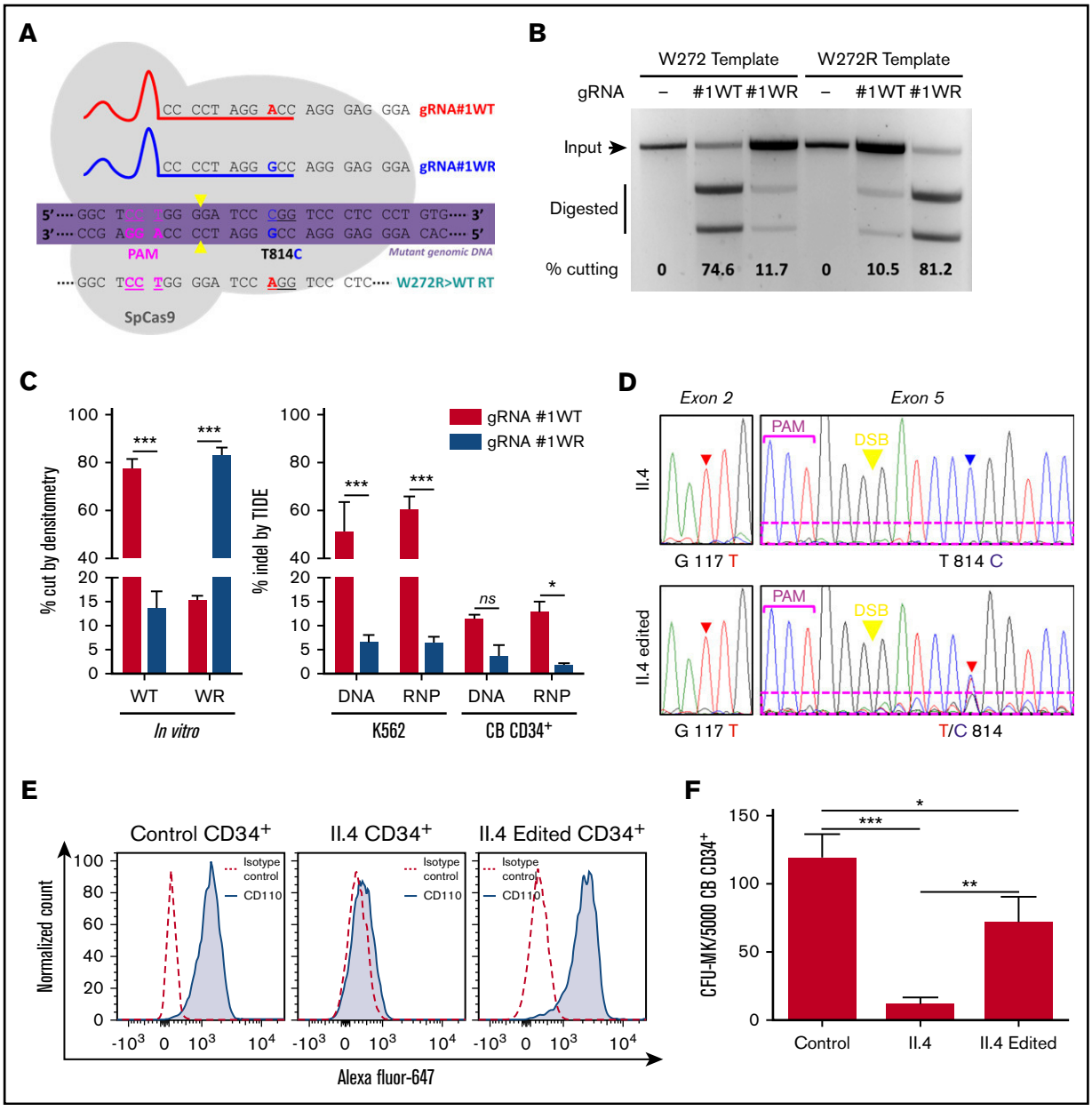

Figure 4. Gene editing in K562 cells and primary CD34$^+$ cells. (A) Schematic of sequence-specific gRNA#1WT and gRNA#1WR and their target genomic *MPL* sequence representing the protospacer-adjacent motif (PAM), double-strand break site (yellow arrowheads), and the W272R single point mutation site (T814C). DNA codons are underlined, and the repair template (RT) used to convert the W272R mutation to the WT sequence (W272R>WT) is also represented. (B) Example of in vitro digestion assay with gRNA#1WT or gRNA#1WR in the presence of their match or mismatch target sequences. Quantification of cutting efficiency was performed by using densitometry analysis. (C) Left panel shows quantification of in vitro cutting capabilities of gRNA#1WT and gRNA#1WR. Right panel shows quantification of the percentage of indel formation obtained with gRNA#1WT and gRNA#1WR when delivered as plasmid DNA or RNP complexes in K562 or CB CD34$^+$ cells. (D) Control, unedited, and edited CD34$^+$ cells isolated from patient II.4 were sequenced at day 5 after editing. G117T represents the K39N mutation and T814C represents the W272R mutation. Dotted magenta rectangles highlight the presence of additional overlapping sequences in edited cells for the T814C locus, indicating an off-target effect. (E) Flow cytometry analysis of anti-Mpl (CD110)-AlexaFluor-647 binding on control CD34$^+$ cells, unedited patient II.4 CD34$^+$ cells, or edited II.4 CD34$^+$ cells at day 5 after editing. (F) In vitro megakaryocytic colony formation assay conducted in the presence of Tpo with the same cell samples used in panel E. *$P < .05$; **$P < .005$; ***$P < .0001$. CFU, colony-forming unit; DSB, double-strand break.

good and similar cutting efficiency nearing 80% (quantified in Figure 4C, left panel). However, we also noticed that both gRNAs were able to cut their mismatch target to a nonnegligible level (about 15%), indicating the potential for a strong off-target effect of gRNA#1WR on the WT *MPL* sequence if it is used in a heterozygous cell-editing setup.

We then compared 2 different CRISPR-Cas9 delivery methods (plasmid DNA and RNPs) to edit K562 cells and HD CB CD34$^+$ cells. In both cases, we used electroporation to deliver either 0.5 μg of plasmid DNA or 200 pmol of RNPs per 200 000 cells. Editing efficiency was measured at 24 to 48 hours

Frame shift	Sequence (amplicon sequencing by NGS)	Indels	Repair
Ref.	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGATCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	Wildtype	
Ref.	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGATCCCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	Mutant	
Most abundant amplicons			
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGATCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG		HDR
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGATCCCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG		
Additional amplicons detected (potentially restorative sequences are indicated in green)			
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGCTCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	silent	HDR + NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCT---------GGTCCCTCCCTGTGACTGTGGACCTGCCTGG	9 bp del	NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGT---------GGATCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	9 bp del	HDR + NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCT-CT----------GTCCCTCCCTGTGACTGTGGACCTGCCTGG	10 bp del	NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTC-----GACCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	5 bp del	HDR + NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTTCT-GGGATCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	1 bp del	HDR + NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGACCCTGGTCCCTCCCTGTGCCTGTGGACCTGCCTGG	mutation	HDR + NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCT---GATCCCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	3 bp del	NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGT---------GGATCCCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	9 bp del	NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGCTCCCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	silent	NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGACCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	mutation	HDR + NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGG---------CCCTCCCTGTGACTGTGGACCTGCCTGG	9 bp del	NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGGATCCTGGCCCCTCCCTGTGACTGTGGACCTGCCTGG	mutation	HDR + NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCT-GGGACCCCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	1 bp del	NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCT-GGGACCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	1 bp del	HDR + NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGGG-----GGTCCCTCCCTGTGACTGTGGACCTGCCTGG	5 bp del	NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTC--------CCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	8 bp del	HDR + NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTC--G----T---GGTCCCTCCCTGTGACTGTGGACCTGCCTGG	9 bp del	NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCT------GATCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	6 bp del	HDR + NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCT--GGATCCCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	2 bp del	NHEJ
yes	CTGCGCAGCGAACCTGATGGGATC----------CCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	23 bp del	NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGT-------GGGGATCCCGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	7 bp del	NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCT---GATCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	3 bp del	HDR + NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCT----------CTGTGACTGTGGACCTGCCTGG	21 bp del	NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGT----------GATCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	10 bp del	HDR + NHEJ
yes	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCT--T----------TGGACCTGCCTGG	29 bp del	NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCC------TCCTGGTCCCTCCCTGTGACTGTGGACCTGCCTGG	6 bp del	HDR + NHEJ
no	CTGCGCAGCGAACCTGATGGGATCTCCCTCGGTGGCTCCTGGG---------CCCTCCCTGTGACTGTGGACCTGCCTGG	9 bp del	NHEJ

Figure 5. Next-generation sequencing of *MPL* exon 5 PCR amplicons after gene editing. Amplicons generated from gDNA obtained from patient II.4 edited CD34$^+$ cells were subjected to next-generation sequencing. Sequences, other than properly HDR edited or unedited, that can potentially yield functional Mpl proteins are indicated in green.

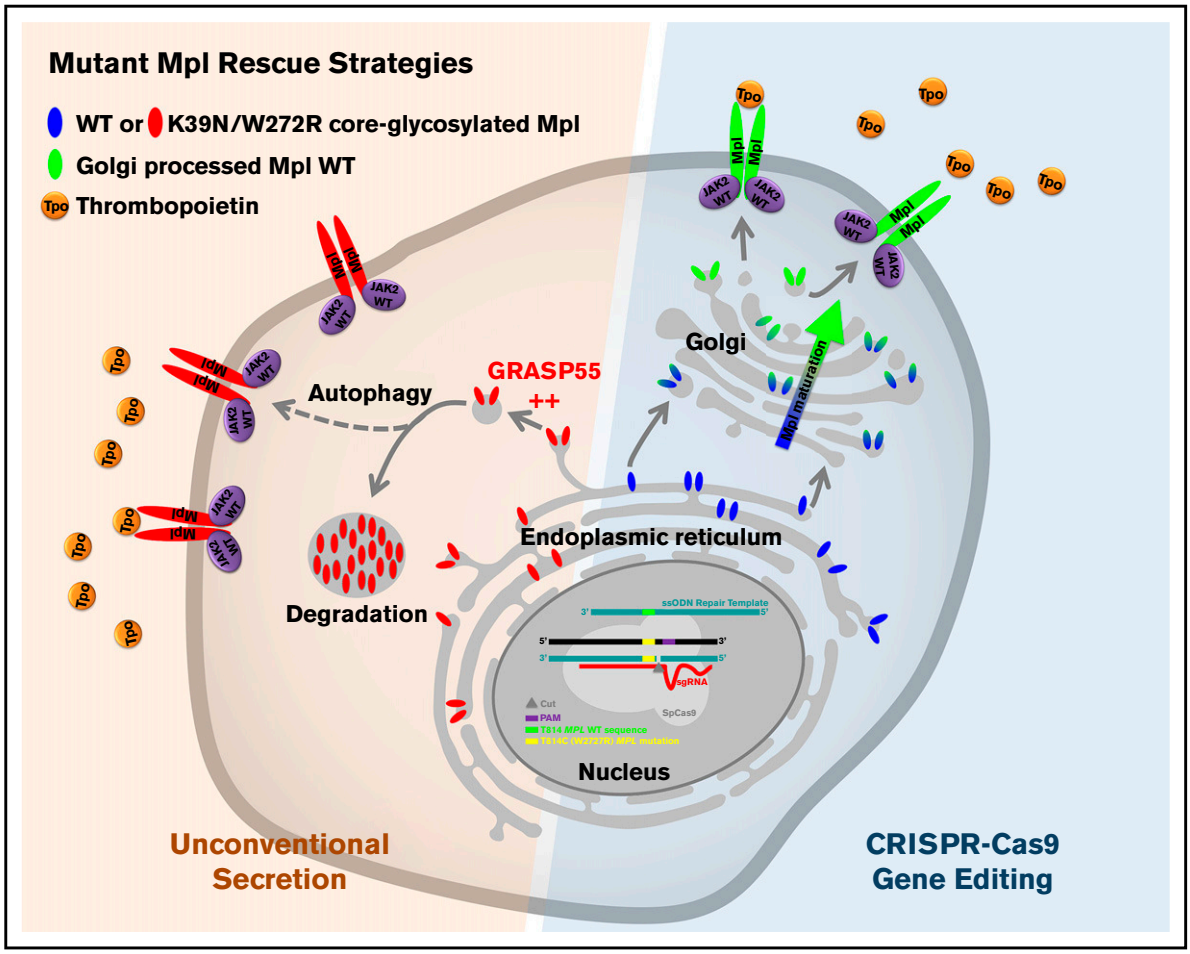

Figure 6. Functional rescue strategies for Mpl mutants. Schematic summary of the 2 rescue approaches used to restore Mpl function: (1) overexpression of GRASP55 to force immature Mpl receptor expression at the cell surface using unconventional autophagy-dependent secretion and (2) CRISPR-Cas9 gene editing to convert mutated Mpl DNA sequence to WT sequence. sgRNA, single-guide RNA.

posttransfection by using the tracking of indels by decomposition (TIDE) Web tool. This tool performs Sanger sequencing trace file deconvolution to measure the percentage of indel formation after nonhomologous end joining repair of a Cas9-induced double-strand break. Figure 4C (right panel) shows that gRNA#1WT performed equally well when delivered as either a plasmid DNA or an RNP in K562 cells. Both delivery systems were also found comparable in inducing indel formation when editing CB CD34$^+$ cells, with a four- to sixfold decrease in efficiency compared with editing in K562 cells (~50% and ~10% of indels, respectively). As noted in the in vitro cutting assay, a significant 2% to 9% of off-target cutting was measured for gRNA#1WR when delivered to WT *MPL* cells.

Gene editing rescues double K39N/W272R Mpl mutant function in primary hematopoietic CD34$^+$ cells

Our gene editing approach was next applied to primary HSCs isolated from the umbilical CB of patient II.4. After thawing, 60 000 CD34$^+$ cells homozygous for the double K39N/W272R Mpl mutation were obtained from 104 $\times$ 10^6 CB mononuclear cells (0.058%), consistent with a CAMT type I diagnostic. Twenty-four hours after isolation, two-thirds of the cells were subjected to gene editing using 40 pmol of gRNA#1WR/Cas9 RNPs and the WR>WT repair template. Parental and edited cells were then maintained in CD34$^+$ expansion media, containing (among other cytokines) 100 ng/mL of Tpo for 5 days before being used in functional assays to assess editing efficiency. Figure 4D shows the sequencing results of unedited (top panels) and edited (lower panels) cells at day 5 after editing. The homozygous K39N mutation could be found in both populations, as expected. However, in the edited cell population, the W272R mutation seemed to be mostly heterozygous. We concluded that most of the edited cells contained only 1 modified copy of *MPL*, reverting the sequences from R272 to W272. It is also interesting to note the presence of additional sequencing traces in the lower right panel of Figure 4D compared with the upper right panel (magenta rectangle), indicating off-target effects of gRNA#1WR being able to cut a previously edited sequence. This likely reflects the use of nonhomologous end joining (NHEJ) instead of HDR editing as a repair mechanism by the cell machinery. To determine the extent to which other repair mechanisms could generate restorative sequences in the *MPL* locus, we performed amplicon sequencing by next-generation sequencing. Data in Figure 5 indicate that several alternatively edited alleles, obtained by NHEJ

only or a combination of HDR editing and NHEJ, are capable of encoding functional Mpl proteins. These alternate alleles are composed mostly of in-frame deletions of the mutant residue.

We used 2 different assays to show that only 1 functional allele was required for edited cells to deliver functional Mpl to the cell surface and proliferate in the presence of Tpo, regardless of whether the repair was obtained through the HDR editing or the NHEJ mechanism. Receptors on the surface of edited cells were detected by using a fluorescently labeled anti-Mpl antibody and were quantified by flow cytometry. As shown in Figure 4E, control $CD34^+$ and II.4 edited $CD34^+$ cells displayed similar amounts of surface Mpl proteins, whereas unedited II.4 $CD34^+$ cells did not bind Mpl antibodies. We specifically selected for cells with surface expression of Mpl by maintaining cultures before and after editing in high Tpo conditions (100 ng/mL) as the sole growth factor. Finally, single-colony assay results (Figure 4F) demonstrated that edited $CD34^+$ cells from patient II.4 were able to generate a significantly higher number of megakaryocytic colonies in the presence of Tpo than unedited cells from this patient and compared with control CB $CD34^+$ cells. Results observed in this experiment reflect the presence of both efficiently edited cells and cells bearing alternate *MPL* exon 5 sequences that restored functionality. We speculate that alternative exon 5 rearrangements may couple in cis with the activating K39N mutation, which provides edited cells with a proliferation advantage superior to that of unedited $CD34^+$ cells. Growth curves of cell lines that express Mpl K39N support this hypothesis (Figure 3B-C).

Discussion

To the best of our knowledge, this is the first report of a double mutation in *MPL* (G117T/T814C) that results in intracellular retention of Mpl receptors in the ER and the first study to fully characterize the pathogenic mechanism behind the association of this mutation with CAMT. In this CAMT type I family, the Mpl W272R mutation was present in cis with the activated K39N variant associated with hereditary thrombocytosis. We show that the trafficking defect of the Mpl W272R receptor effectively prevents surface expression and thus Tpo-mediated signaling. Although receptors bearing only the K39N mutation are also partially retained in the ER, a fraction of this hyperactive form of Mpl does reach the cell surface. Hence the absence of thrombocytosis for the 3 family members bearing the Mpl K39N polymorphism in a heterozygous fashion can be explained by the in cis loss-of-function W272R mutation and 1 normal copy of the *MPL* gene. Other combinations of the W272R mutation with another in cis genetic alteration in a heterozygous setting would likely give rise to a similar phenotype because the W272R mutation is responsible for the Mpl trafficking defects. For the 3 children who were homozygous for Mpl K39N/W272R, the W272R mutation overrides the activating K39N mutation because the Mpl receptors are retained in the ER and cannot respond to extracellular ligand. Consistent with reports of defective feedback regulation of circulating Tpo in CAMT,[25] family members who are homozygous for the *MPL* K39N/W272R mutant presented with highly elevated Tpo levels in blood and a deficit in 2 hematopoietic lineages. The single point mutation T814C of *MPL* has recently been identified in a database generated by whole-exome sequencing of gastric cancer patients,[26] indicating that additional cases may occur.

Importantly, we were able to design and test several strategies to rescue Mpl receptor trafficking and function (summarized in Figure 6). Like the most common CFTR mutation (DeltaF508) in cystic fibrosis,[27] the doubly mutated Mpl protein retains partial functionality. GRASP55 overexpression enabled Mpl K39N/W272R to reach the surface by the autophagy secretory pathway[22] where it could bind Tpo and trigger JAK-STAT–mediated signaling (Figure 3A). This approach may eventually be translated into patient treatment now that recent drug screening studies have identified compounds that activate autophagy.[28] We also demonstrated that CRISPR-Cas9 gene-editing methods can be used to repair a disease-causing mutation in the Mpl coding sequence. We showed that transfected cell lines expressing mutant Mpl were efficiently edited to restore Mpl WT sequence trafficking to the cell surface and response to Tpo. Because CAMT patients typically have high levels of circulating Tpo,[25] even a partial recovery of Mpl surface expression might normalize hematopoiesis or, at a minimum, provide additional time to match donors for a bone marrow transplantation.

We also applied CRISPR-Cas9 to primary CB-derived hematopoietic cells from study patient II.4 and were able to partially restore WT *MPL* sequence. Edited cells displayed a normal surface expression of the receptor and could generate in vitro megakaryocytic colonies. Despite a mutation-specific design of gRNA#1WR, we could see significant amounts of off-target effect in edited cells. A silent mutation of the PAM sequence from NGG to NTG,[29] carried by the ssODN repair template, would likely circumvent this issue, although without allowing a truly scarless editing strategy. In addition, we found that alternate genomic sequences, potentially restorative of Mpl function, were also induced by our gene editing strategy. Some of these sequences resulted from a combination of HDR editing and NHEJ, whereas others were the result of NHEJ alone. This is an important finding, which indicates that alternate editing strategies can be developed and refined to restore Mpl function. Hence, our study provides in vitro proof-of-principle that *MPL* mutations detected in CAMT patients may be corrected by modern gene engineering methods and/or by autophagy-activating drugs. The promise of gene therapy for CAMT and other hereditary hematologic disorders awaits successes in the rapidly advancing field of genome editing of patient-derived stem cells.[17,30] Because only a subset of hematopoietic progenitor cells may need to be edited to achieve long-term polyclonal hematopoiesis, this approach could represent a cure for the majority of CAMT patients.

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Authorship

Contribution: E.J. and T.L.-B. enrolled study patients; C.C., R.G., E.H.C., and S.C. performed experiments; C.C., R.G., S.C., and B.S.W. analyzed results and created the figures; C.C. and B.S.W. designed the research; and C.C., S.H., and B.S.W. wrote the paper with contributions from all authors.

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References

1. Ihara K, Ishii E, Eguchi M, et al. Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. *Proc Natl Acad Sci USA*. 1999;96(6):3132-3136.
2. Bennett M, Stroncek DF. Recent advances in the bcr-abl negative chronic myeloproliferative diseases. *J Transl Med*. 2006;4:41.
3. He X, Chen Z, Jiang Y, Qiu X, Zhao X. Different mutations of the human c-mpl gene indicate distinct haematopoietic diseases. *J Hematol Oncol*. 2013;6:11.
4. Tijssen MR, di Summa F, van den Oudenrijn S, et al. Functional analysis of single amino-acid mutations in the thrombopoietin-receptor Mpl underlying congenital amegakaryocytic thrombocytopenia. *Br J Haematol*. 2008;141(6):808-813.
5. Varghese LN, Zhang JG, Young SN, et al. Functional characterization of c-Mpl ectodomain mutations that underlie congenital amegakaryocytic thrombocytopenia. *Growth Factors*. 2014;32(1):18-26.
6. Stockklausner C, Klotter AC, Dickemann N, et al. The thrombopoietin receptor P106L mutation functionally separates receptor signaling activity from thrombopoietin homeostasis. *Blood*. 2015;125(7):1159-1169.
7. Moliterno AR, Williams DM, Gutierrez-Alamillo LI, Salvatori R, Ingersoll RG, Spivak JL. Mpl Baltimore: a thrombopoietin receptor polymorphism associated with thrombocytosis. *Proc Natl Acad Sci USA*. 2004;101(31):11444-11447.
8. Han L, Schubert C, Köhler J, et al. Calreticulin-mutant proteins induce megakaryocytic signaling to transform hematopoietic cells and undergo accelerated degradation and Golgi-mediated secretion. *J Hematol Oncol*. 2016;9(1):45.
9. Chachoua I, Pecquet C, El-Khoury M, et al. Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. *Blood*. 2016;127(10):1325-1335.
10. Lu X, Levine R, Tong W, et al. Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. *Proc Natl Acad Sci USA*. 2005;102(52):18962-18967.
11. Cleyrat C, Vilaine M, Boissinot M, et al. Surface expression of Mpl: aberrant Mpl trafficking in myeloproliferafive neoplasms is linked to insufficient expression of wild type Jak2 [abstract]. *Blood*. 2011;118(21). Abstract 2820.
12. Tong W, Sulahian R, Gross AW, Hendon N, Lodish HF, Huang LJ. The membrane-proximal region of the thrombopoietin receptor confers its high surface expression by JAK2-dependent and -independent mechanisms. *J Biol Chem*. 2006;281(50):38930-38940.
13. Cleyrat C, Darehshouri A, Steinkamp MP, et al. Mpl traffics to the cell surface through conventional and unconventional routes. *Traffic*. 2014;15(9):961-982.
14. Germeshausen M, Ballmaier M, Welte K. MPL mutations in 23 patients suffering from congenital amegakaryocytic thrombocytopenia: the type of mutation predicts the course of the disease. *Hum Mutat*. 2006;27(3):296.
15. Ballmaier M, Germeshausen M. Congenital amegakaryocytic thrombocytopenia: clinical presentation, diagnosis, and treatment. *Semin Thromb Hemost*. 2011;37(6):673-681.
16. Mahadeo KM, Tewari P, Parikh SH, et al. Durable engraftment and correction of hematological abnormalities in children with congenital amegakaryocytic thrombocytopenia following myeloablative umbilical cord blood transplantation. *Pediatr Transplant*. 2015;19(7):753-757.
17. Shui B, Hernandez Matias L, Guo Y, Peng Y. The rise of CRISPR/Cas for genome editing in stem cells. *Stem Cells Int*. 2016;2016:8140168.
18. DeWitt MA, Magis W, Bray NL, et al. Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med*. 2016;8(360):360ra134.
19. Dobo I, Boiret N, Lippert E, et al. A standardized endogenous megakaryocytic erythroid colony assay for the diagnosis of essential thrombocythemia. *Haematologica*. 2004;89(10):1207-1212.
20. Chen WM, Yu B, Zhang Q, Xu P. Identification of the residues in the extracellular domain of thrombopoietin receptor involved in the binding of thrombopoietin and a nuclear distribution protein (human NUDC). *J Biol Chem*. 2010;285(34):26697-26709.
21. Komatsu N, Kunitama M, Yamada M, et al. Establishment and characterization of the thrombopoietin-dependent megakaryocytic cell line, UT-7/TPO. *Blood*. 1996;87(11):4552-4560.
22. Ponpuak M, Mandell MA, Kimura T, Chauhan S, Cleyrat C, Deretic V. Secretory autophagy. *Curr Opin Cell Biol*. 2015;35:106-116.
23. Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat Biotechnol*. 2016;34(3):339-344.

24. Cradick TJ, Fine EJ, Antico CJ, Bao G. CRISPR/Cas9 systems targeting β-globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Res*. 2013;41(20):9584-9592.

25. Al-Qahtani FS. Congenital amegakaryocytic thrombocytopenia: a brief review of the literature. *Clin Med Insights Pathol*. 2010;3:25-30.

26. Kim TM, Jung SH, Kim MS, et al. The mutational burdens and evolutionary ages of early gastric cancers are comparable to those of advanced gastric cancers. *J Pathol*. 2014;234(3):365-374.

27. Gee HY, Noh SH, Tang BL, Kim KH, Lee MG. Rescue of ΔF508-CFTR trafficking via a GRASP-dependent unconventional secretion pathway. *Cell*. 2011;146(5):746-760.

28. Chauhan S, Ahmed Z, Bradfute SB, et al. Pharmaceutical screen identifies novel target processes for activation of autophagy with a broad translational potential. *Nat Commun*. 2015;6:8620.

29. Hsu PD, Scott DA, Weinstein JA, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol*. 2013;31(9):827-832.

30. Tasan I, Jain S, Zhao H. Use of genome-editing tools to treat sickle cell disease. *Hum Genet*. 2016;135(9):1011-1028.